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FACULDADE DE FARMÁCIA
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Valorização de germinados de *Brassica oleracea* através da avaliação nutricional e da composição em compostos bioativos

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Porto, 2014

Tese de doutoramento

Valorização de germinados de *Brassica oleracea* através da avaliação nutricional e da composição em compostos bioativos

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"Talvez não tenhamos conseguido fazer o melhor, mas lutamos para que o melhor fosse feito. Não somos o que deveríamos ser, não somos o que iremos ser... Mas Graças a Deus, não somos o que éramos."

Marthin Luther King

Resumo

A importância do consumo de vegetais frescos é reconhecida mundialmente como um dos meios mais eficazes para preservar e melhorar a saúde. Para isso contribui o seu elevado teor em compostos bioativos, importante para a melhoria ou manutenção de uma melhor condição de saúde (ex.: fitoquímicos com ação anticancerígena e antioxidante). Todavia, é também do conhecimento público, que o consumo de vegetais nas sociedades do Ocidente fica muito aquém dos valores recomendados pelas organizações mundiais de saúde (FAO e WHO recomendam um consumo aproximado de 400g/vegetais por dia). Os consumidores alegam muitas vezes a falta de conveniência para evitar ou "esquecer" de incluir vegetais nas suas dietas. Contudo, existe também uma crescente preocupação com a adoção de um estilo de vida saudável, tendo a procura de alimentos convenientes e saudáveis aumentado nos últimos anos. É neste contexto que surge um maior interesse pelos vegetais minimamente processados, prontos a consumir. Este é, por isso, um mercado em constante evolução para corresponder à demanda dos consumidores. Um dos produtos deste tipo, que tem recebido uma grande aceitação pelos consumidores, são os germinados, já muito comuns nos mercados asiáticos e que começam agora a conquistar os consumidores ocidentais devido às suas propriedades nutritivas e de conveniência.

O processo de produção de germinados é simples e de baixo custo, e consiste na imersão das sementes até à protrusão do sistema radicular da semente. O processo de germinação de sementes é também associado a um aumento do valor nutricional em relação à composição da semente, devido à ativação do metabolismo que promove a hidrólise de proteínas, hidratos de carbono e a síntese e acumulação de novos compostos bioativos. Este processo de acumulação de novos compostos durante a germinação faz que com os germinados sejam considerados como um alimento funcional, uma vez que alguns germinados podem apresentar um teor de bioativos até dez vezes superior ao teor encontrado no mesmo vegetal completamente maturo. Contudo, a informação genética presente nas sementes e as condições ambientais durante a germinação vão influenciar significativamente a composição final dos germinados, sendo necessário otimizar o processo para cada espécie e/ou variedade, para potenciar as qualidade nutricionais do produto final. No mercado atual encontram-se principalmente germinados de algumas espécies de leguminosas. Contudo, o interesse nos germinados de espécies da família das brássicas tem aumentado, devido ao elevado valor económico e nutricional que está associado aos vegetais desta família.

O objetivo principal do estudo desenvolvido nesta tese foi a determinação das melhores condições ambientais para a produção de germinados, com maior valor

nutricional, de quatro variedades de *Brassicae oleracea*. Duas das variedades estudadas são das mais consumidas mundialmente, a Couve Roxa e o Brócolo, sendo as outras duas, variedades tradicionais e muito comuns do Norte de Portugal, a Couve-galega e a Couve-Penca. Particularmente, o estudo focou a influência de determinadas condições ambientais, nomeadamente o fotoperíodo e o período de germinação, na composição nutricional (ex: proteína, fibra dietética, gordura, perfil de aminoácidos e de ácidos gordos), no teor de compostos bioativos (compostos fenólicos, glucosinolatos e ácidos orgânicos) e na potencial ação antioxidante e antimicrobiana dos germinados ao longo da germinação. Foi também estudada a estabilidade de diferentes compostos bioativos e a qualidade microbiológica dos germinados ao longo de um período de armazenamento refrigerado, simulando as condições a que o produto é submetido até chegar ao consumidor.

Os germinados estudados revelaram ser uma excelente fonte de proteína e fibra dietética. O elevado teor de selénio deste produto foi uma das características que mais se destacou da sua composição mineral, bem como a sua equilibrada composição em aminoácidos. O seu perfil de glucosinolatos mostrou uma grande predominância de compostos alifáticos como a sinigrina e a glucorafanina, reconhecidos pela sua potencial ação anticancerígena. Os perfis de aminoácidos e de ácidos gordos encontrados mostraram ser fortemente influenciados pela exposição à luz durante a germinação, sendo potenciados pelo uso de uma germinação sem luz. Já no que se refere à potencial ação antioxidante dos germinados, esta apresentou valores superiores quando os germinados eram expostos a ciclos de luz/escuro. Este foi também o fotoperíodo que mais potenciou o teor de glucosinolatos e de alguns ácidos orgânicos encontrados nas variedades estudadas. Relativamente a uma potencial ação antimicrobiana, os germinados mostraram uma ação significativa contra alguns dos microrganismos patogénicos mais preocupantes em termos de segurança alimentar, apresentando essa atividade uma boa correlação com o teor de ácidos orgânicos presentes nas amostras. Quanto ao período de germinação, na maioria dos compostos estudados, o uso de períodos de germinação mais curtos (entre 7 e 9 dias) originou a presença de um maior teor de compostos bioativos. Relativamente à estabilidade da qualidade nutricional e microbiológica dos germinados, os resultados obtidos apontaram para uma maior preservação da qualidade durante os primeiros 7 dias após a colheita. Contudo, a perda de compostos bioativos foi maior nos germinados com exposição à luz, apresentando os germinados produzidos na ausência da luz uma maior preservação de compostos fenólicos e glucosinolatos. A qualidade microbiológica destes produtos não foi afetada pelas condições ambientais durante a germinação, não sendo encontrado nenhum micorganismo patogénico durante o período de armazenamento.

Palavras-chave: germinados, qualidade nutricional, atividade antioxidante, glucosinolatos, compostos fenólicos, ácidos orgânicos, atividade antimicrobiana, armazenamento

Abstract

The importance of eating fresh vegetables is recognized worldwide as one of the most effective ways to preserve and improve health. The beneficial health effects can be attributed to the high content of bioactive compounds found in fresh-cut vegetables (eg. phytochemicals with antioxidant and anticancer activity). However, it is also widely known that the level of vegetable intake in Western societies is far below that recommended by world health organisations (FAO and WHO recommend a minimum vegetable intake of about 400g/day). Whereas some are claiming a lack of convenience as an excuse to avoid or "forget" to include vegetables in their diet, others are starting to adopt healthier lifestyles, as suggested by recent increases in consumer demand for convenience and healthy foods. It is in this context that a growing interest for minimally processed ready-to-eat vegetables is beginning to show. Therefore, this is a market in constant evolution to meet consumer demand. Sprouts, which are an example of this type of product and very common in Asian markets, are beginning to receiving wide acceptance by Western consumers due to its nutritional and convenience properties.

The sprouts production process is simple and inexpensive, consisting of the immersion of seeds up to the protrusion of the roots of the seed. The process of seed sprouting is also associated with an increased nutritional value when compared to the composition of the seed, due to metabolic activation which promotes the hydrolysis of proteins, carbohydrates and the synthesis and accumulation of new bioactive compounds. This process of accumulating new compounds during germination causes sprouts to be considered as functional food, due to the fact that some sprouts may have up to ten times higher the content of bioactive compounds found in those mature plants. It is, however, necessary to optimize the process for each type and / or variety to enhance the nutritional quality of the final product, as the genetic information and the environmental conditions during germination will significantly influence the final composition of the sprouted seeds. Presently, it is possible to find sprouts of some legume species in most markets and interest in sprouted species of the Brassicaceae family is increasing due to the high economic and nutritional value associated with plants of this family.

The main objective of the present study was to identify the best environmental conditions for the production of four varieties of Brassica oleracea sprouts, with high nutritional value. Two of the studied varieties, red cabbage and broccoli, are within the most consumed in the world and, the other two Kales (galega kale and penca cabbage), are traditional varieties, very common in northern Portugal. Particularly, this study focused on the influence of certain environmental conditions, such as the photoperiod and the

germination period, in the nutritional composition (e.g. protein, dietary fibre, fat, amino acid and fatty acid profile); in the content of bioactive compounds (compounds phenolics, glucosinolates and organic acids); in the potential of both antioxidant and antimicrobial activity of the sprouts throughout germination. In addition, the stability of different bioactive compounds and the microbiological quality of the germinated seeds over a period of cold storage, simulating the conditions to which the product is subjected to reach the consumer, were also assessed.

The studied sprouts proved to be an excellent source of protein and dietary fibre. The high selenium content of this product was one of the features that stood out from its mineral composition as did its balanced amino acid composition. The glucosinolate profile showed a great predominance of aliphatic compounds such as sinigrin and glucoraphanin, known for their potential anticancer activity. The amino acid and the fatty acids profiles found were strongly influenced by exposure to light during germination, being enhanced by the germination in dark conditions. Regarding the potential antioxidant of the sprouts, it showed higher values when exposed to light / dark cycles. This was also the photoperiod that potentiate the glucosinolate content and the organic acids found in the studied varieties. Regarding the potential antimicrobial activity, sprouts showed significant action against some foodborne pathogens of most concern in terms of food safety, revealing a good correlation with the organic acids content of samples. As for the germination period, in most of studied compounds, the use of shorter periods of germination (between 7 and 9 days) originated a higher content of bioactive compounds. For the stability of the nutritional and microbiological quality of the sprouts, the results pointed to a higher maintenance of quality during the first 7 days after harvesting. The loss of bioactive compounds was, however, higher for sprouts produced under light conditions than for sprouts grown in dark, having the latter ones showed a greater preservation of glucosinolates and phenolic compounds. The microbiological quality of these products was not affected by the environmental conditions during germination as no pathogens were found during the storage period.

Keywords: sprouts, nutritional quality, antioxidant activity, glucosinolates, phenolic compounds, organic acids, antimicrobial activity, storage

Lista de publicações

Publicações de artigos em revistas de circulação internacional com arbitragem científica referenciadas no Journal Citation Reports da ISI Web of Knowledge:

1. Ana Paula Vale, Honorina Cidade, Madalena Pinto, M.Beatriz P.P. Oliveira, 2014. Effect of sprouting and light cycle on antioxidant activity of *Brassica oleracea* varieties. *Food Chemistry, 165*(0), 379-387.

2. A.P. Vale, J. Santos, N.V. Brito, V.Peixoto, Rosa, Carvalho, M.Beatriz P.P.Oliveira. Light influence in the nutritional composition of *Brassica oleracea* sprouts. *Food Chemistry*. Submited.

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Comunicações orais

Vale, A.P., Brito, N.V. Cidade, H.M., Oliveira M. B. P.P. 2011. Influência do tempo de germinação na atividade antioxidante e no teor de compostos fenólicos de germinados de *Brassica oleracea*. XXII Encontro Nacional da Sociedade Portuguesa de Química/XXIIENSPQ, Braga, Portugal.

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Comunicações em poster em encontros científicos nacionais:

Vale, A.P., Melia, N., Campos, S., Oliveira, M.B.P.P. 2012. Avaliação da composição de ácidos gordos de germinados de *Brassica oleracea*. 11º Encontro de Química dos Alimentos. Qualidade dos Alimentos: Novos desafios. 16-19 de Setembro, 2012, Bragança. pp 54 (CP8)

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Lista de Abreviaturas

AA – Free Amino Acids

AAPH - 2,2'-Azobis(2-amidinopropane) dihydrochloride

ABAP - 2,2' -azobis(2-amidinopropane)

ALA – α-Linolenic Acid

ANOVA - Analysis of variance

AOAC – Association of Analytical Communities

BPW – Buffered Peptone Water

Bras-EDB - Base de Dados Europeia de *Brassica*

cfu - Colony forming unit

DAD – Diode Array Detector

DPPH – 1,1-diphenyl-2-picrylhydrazyl

EAA – Essential Amino Acid

EDTA - Ethylenediaminetetraacetic acid

EFSA – European Food Safety Authority

EUA – Estados Unidos da América

FA – Fatty Acid

FAME – Fatty Acid Methil Esters

FAO - Food and Agriculture Organization

FID – Flame Ionization Detector

FSAI – Food Safety Authority of Ireland

GL – Glucosinolate

GS – Green Sprouts

HPLC – High Performance Liquid Chromatography

HPLC DAD - High-Performance Liquid Chromatography with Diode-Array Detection

HUFA – Highly Unsatureted Fatty Acid

IC_{50} - quantidade de substância antioxidante necessária para reduzir em 50% a concentração inicial de radicais livres

ITC – Isothiocyanato

kGy – Quilogray

kHz - Quilohertz

LA – Linoleic Acid

LDA – Linear Discriminant Analisys

LSD – Least Significant Difference

MIC – Minimal Inibitory Concentration

MIC ^{int} – Minimal Inibitory Concentration 2-p-iodophenyl-3-p-nitrophenyl-5-phenyl tetrazolium chloride

MPa - Megapascal

MUFA – Monounsatureted Fatty Acid

n.d. - not detected

NAC-MCF – National Advisory Committee on Microbiological Criteria for Food

NEAA – Non Essential Amino Acid

NFE – Nitrogen-free Extract

nm – nanometros

OMS – Organização Mundial de Saúde

PCA – Principal component analysis

PFCA – Portuguese Food Composition Table

pH - Hydrogen ion potential

ppm – Partes por milhão

PUFA - Polyunsatureted Fatty Acid

PVDF - Polyvinylidene fluoride

PW – Peptone water

r – Correlation coefficient

R^2 – Coeficiente de determinação

RBCA – Rose-Bengal Chloramphenicol Agar

RDA – Recommended Dietary Allowances

ROO - Alkyl Peroxyl Radical

ROS – Reactive Oxygen Species

RUS – Salmonela Enrichment Broth

SDS – Diethyl Agar

SE – Standard Error

SFA – Satureted Fatty Acid

TDF – Total Dietary Fiber

TFC – Total Flavenoid Content

TPC – Total Phenolic Content

TSA – Triptic Soy Agar

TT – Selective Enrichment Broth

ufc – unidade formadora de colonias

USFA – Ultra Unsatureted Fatty Acid

USFDA – United States Food and Drugs Administration

UV - Ultraviolet radiation

UV-vis - Ultraviolet radiation visible

v/v - volume/volume

VRVGA – Violet-Red Agar

vs – Versus

WHO – World Health Organization

WS – Total Darkness

XLD – Xylose Lysine Deoxycholate Agar

CAPITULO 1.

Motivação, Objetivos, Organização e Estrutura da Tese

1.1 Motivação para a dissertação

A produção de germinados é o resultado do processo de germinação de sementes, e é uma prática milenar em muitos países asiáticos. A primeira referência sobre alimentos germinados remonta ao ano 3000 a.C., na China. No Ocidente, observou-se nas últimas décadas uma intensa expansão do consumo deste tipo de alimentos, fenómeno associado ao seu reconhecido valor nutricional e às alterações no comportamento do consumidor, que tem manifestado uma profunda evolução, particularmente no que diz respeito ao consumo de produtos agroalimentares de origem vegetal. A partir dos anos 90 do século XX, o consumo de saladas e de frutos pré-cortados e embalados, ditos de 4ª gama ou de conveniência, passou a ter grande aceitação pelo consumidor, já que são produtos com um valor acrescentado e estão associados à imagem de produtos frescos e naturais. Atualmente, muitos consumidores procuram nas escolhas alimentares mais do que a simples satisfação de uma necessidade básica, e baseiam as suas escolhas nos benefícios, que podem obter para a saúde, a partir de determinado tipo de alimento.

Neste contexto, a recetividade a novos produtos que propiciem um valor acrescentado, apresenta-se como uma interessante oportunidade de mercado, estando o consumidor recetivo a novas gamas de alimentos, considerados frescos, fáceis de preparar e com elevado valor nutricional. Os germinados fazem parte deste grupo de produtos frescos. São conhecidos como produtos “prontos a consumir” e são considerados uma excelente fonte de aminoácidos, minerais, fibra e compostos fenólicos (1). Os germinados da família *Brassicaceae* são ainda caraterizados por um teor de glucosinolatos e de compostos com propriedades antioxidantes superiores aos da planta adulta (2). Contudo, há ainda desconhecimento sobre a qualidade nutricional dos germinados de diferentes espécies/variedades hortícolas, sendo igualmente necessário proceder à otimização das condições de produção que permitam potenciar as propriedades destes alimentos.

No mercado europeu, como por exemplo na Alemanha, é comercializada uma grande diversidade de germinados de diferentes espécies, destacando-se o feijão azuki (*Phaseolus angularis*), luzerna (*Medicago sativa*), brocolo (*Brassica oleracea* convar. *botrytis*), agrião (*Lepidium sativum*), lentilha (*Lens culinaris*), feijão mungo (*Phaseolus aureus*), mostarda branca (*Sinapis alba*), ervilha verde e amarela (*Pisum sativum*), cebola (*Allium cepa*), rabanete (*Raphanus sativus*), arroz (*Oryza sativa* L.), centeio (*Secale cereale*), sésamo (*Sesamum indicum*), girassol (*Helianthus annuus*) e trigo (*Triticum aestivum*) (3).

Em Portugal, são produzidos e comercializados germinados de um reduzido número de espécies, sobretudo de soja. Impõe-se desenvolver e demonstrar a potencial utilização (produção e consumo) de germinados de espécies já comercializadas no exterior, mas também de algumas variedades hortícolas, nomeadamente de couves portuguesas.

Estas variedades fazem parte da dieta dos portugueses e a sua apresentação como germinados poderá ser uma mais-valia para os produtores, comerciantes e para o próprio consumidor. Além disso, a sua utilização permite ainda valorizar as espécies hortícolas regionais, que têm vindo a ser desvalorizadas devido à pressão exercida pelo mercado de sementes híbridas. Contudo, para que estes produtos recebam a aceitação dos consumidores portugueses é fundamental caracterizá-los em termos de valor nutricional e demonstrar potenciais benefícios inerentes à sua inclusão na dieta alimentar.

1.2. Objetivos

O principal objetivo deste trabalho foi proceder à avaliação nutricional e de compostos bioativos com potenciais efeitos na saúde dos consumidores, de germinados de *Brassica olerácea.* Foram produzidos e analisados germinados de quatro variedades: Brócolo (*B*. *oleracea* L. var. *italica* Plenck, cultivar calabrese), couve-galega (*B. oleracea* var. *acephala* DC), Couve-penca (*B. oleracea* L. var. *costata* DC, landrace Penca da Póvoa) e couve-roxa (*B. oleracea* var. capitata f. rubra).

Para além deste objetivo principal, o presente trabalho decorreu de acordo com um conjunto de objetivos específicos:

a) Estudo do efeito do tempo de germinação e da luz na atividade antioxidante, avaliada *in vitro*;

b) Estudo da influência da luz na qualidade nutricional de germinados (teor de humidade, proteína e aminoácidos livres, fibra bruta e dietética, cinzas e composição mineral, teor de gordura total e perfil de ácidos gordos);

c) Avaliação das condições de germinação e do potencial das variedades de *B.oleracea* para a produção de germinados com elevado teor de glucosinolatos;

d) Avaliação da atividade antimicrobiana de extratos aquosos de germinados e composição fitoquímica dos mesmos (ácidos orgânicos e compostos fenólicos);

e) Estudo do impacto da conservação refrigerada no teor de compostos bioativos e qualidade microbiológica dos germinados.

1.3. Estrutura da Dissertação

A presente dissertação encontra-se estruturada em 8 capítulos diferentes. No presente capítulo é apresentada a motivação para a realização da dissertação, o objetivo geral e os objetivos específicos da investigação realizada, e a forma como se encontra estruturada a dissertação.

No **capítulo 2** faz-se uma abordagem global ao tema da valorização de germinados de sementes. Os fatores que afetam a segurança dos germinados e os principais métodos utilizados para minimizar as contaminações microbiológicas foram especialmente focados. É ainda abordada a qualidade deste tipo de alimentos, numa perspetiva nutricional e nutracêutica. Os principais resultados experimentais obtidos são apresentados nos capítulos 3 e 7, de acordo com a seguinte ordem:

O **capítulo 3** apresenta os resultados dos estudos do potencial antioxidante dos germinados de *B. oleracea*. O delineamento experimental consistiu na avaliação do efeito de diferentes tempos de germinação e de dois tipos de fotoperíodo, sobre a capacidade antioxidante dos germinados. Foram realizados vários estudos *in vitro,* utilizando extratos aquosos de germinados. O potencial antioxidante foi avaliado através de métodos baseados no sequestro de radicais livres: 1,1-difenil-2-picrilidrazil (DPPH), hidroxilo, peroxilo e atividade quelante do ferro tendo por base a redução do Fe^{+3} a Fe^{2+}. O teor de compostos fenólicos e de compostos flavonoides totais foi também determinado.

No **capítulo 4** procedeu-se à avaliação nutricional de germinados produzidos em diferentes condições de fotoperíodo (obscuridade total e ciclos de 16h de luz alternados com 8h de obscuridade). Obtiveram-se assim germinados “brancos” e germinados “verdes”, cujo valor nutricional foi monitorizado mediante a análise de diversos parâmetros nutricionais.

No **capítulo 5** são apresentados e analisados os resultados do perfil de glucosinolatos e a avaliação da atividade da enzima mirosinase, responsável pela degradação destes compostos. A produção de germinados decorreu sob o efeito dos fatores tempo e presença *versus* ausência de luz, fatores estes que exercem influência sobre o teor deste tipo de compostos, procurando-se desta forma otimizar condições para a produção de germinados com elevado teor de glucosinolatos.

No **capítulo 6** apresentam-se os resultados da caracterização de germinados relativamente ao perfil de ácidos orgânicos e aos compostos fenólicos predominantes. Foi ainda avaliado o potencial antimicrobiano de extratos aquosos de germinados, das quatro variedades de *Brassica* em estudo. A atividade antimicrobiana foi estudada utilizando culturas puras de microrganismos Gram positivos e Gram negativos, recorrendo ao método da microdiluição em placa de 96 poços.

O **capítulo 7** é dedicado ao estudo da conservação refrigerada dos germinados e ao impacto do tempo de conservação no teor de compostos fenólicos e no perfil de glucosinolatos. Durante o tempo de conservação refrigerada foi ainda monitorizada a contaminação microbiana dos germinados, uma vez que este é um dos fatores de grande preocupação em matéria de segurança alimentar.

No **capítulo 8** apresentam-se as principais conclusões, os contributos alcançados com esta dissertação e as perspetivas futuras relativamente à produção de germinados.

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CAPÍTULO 2

Germinados de *Brassica oleracea*: Alimentos saudáveis e prontos a consumir

2.1. Introdução

Nas últimas décadas acentuou-se a importância de uma alimentação saudável para a prevenção de doenças crónicas, tais como doenças cardiovasculares e diversos tipos de neoplasias. A alimentação saudável tornou-se o fator determinante e o mais importante para a saúde e bem-estar da população nos países desenvolvidos (1). A Organização Mundial de Saúde (OMS) desenvolveu mesmo, em conjunto com vários países e organizações, orientações alimentares, tendo por objetivo educar a população relativamente a escolhas alimentares saudáveis, políticas alimentares e planos alimentares nutricionalmente equilibrados (2).

Vários estudos demonstraram ainda que o consumo de alimentos de origem vegetal tem efeitos benéficos na prevenção de doenças crónicas (3) e de diversos tipos de cancro (4, 5), sendo o efeito preventivo inerente ao consumo de frutas e de hortícolas, devido à presença de compostos fitoquímicos, tais como os compostos fenólicos (6, 7). Entre os alimentos de origem vegetal com benefícios para a saúde, a família *Brassicaceae* é das mais estudadas ao nível epidemiológico e clínico (8).

A família *Brassicaceae* é constituída por um numeroso grupo de plantas, representando provavelmente um dos grupos de hortícolas de maior consumo na alimentação humana a nível mundial (9). Podem ser plantas anuais, bianuais ou perenes (10) e abarcam cerca de 3000 espécies agrupadas em 350 géneros. Incluem vários tipos de plantas edíveis, entre os quais se destaca o género *Brassica,* que do ponto de vista económico é o mais importante desta família, contendo 37 espécies diferentes (11). A Base de Dados Europeia de *Brassica* (Bras-EDB) inclui 36 coleções de 22 países e possui mais de 19 600 cultivares originais, para mais de 30 espécies de *Brassica*, incluindo espécies cultivadas e espécies silvestres. Entre estas espécies destaca-se a *Brassica oleracea*, a principal espécie de plantas hortícolas, onde se incluem plantas como brócolo, couve-flor, couve penca, couve-galega, couve roxa, couve-de-bruxelas, entre outras (12).

As brássicas são consideradas excelentes fontes de nutrientes e de compostos fitoquímicos com efeitos benéficos sobre a saúde (13). Uma alimentação rica neste tipo de plantas está fortemente associada à redução do risco de desenvolvimento de doenças crónicas graves (14,15,16,17), tais como doenças cardiovasculares e outras doenças degenerativas (18,19,20,21).

O consumo de brássicas na alimentação humana está normalmente associado ao consumo de folhas e de inflorescências. Raramente são utilizadas as sementes, exceto para a extração de óleo (sementes de colza) ou em casos particulares durante o

processamento de alimentos, tais como pão e certo tipo de bolos (22). Contudo, face aos benefícios associados a estas plantas e aos hábitos de consumo (procura de alimentos saudáveis, de fácil preparação), assistiu-se nas últimas décadas a uma intensa investigação na procura de alimentos com caraterísticas funcionais (23) e que respondessem às exigências dos consumidores. Nesse sentido, foi dada especial atenção aos germinados, resultantes da germinação de sementes que, pelo seu elevado valor nutricional se tornaram componentes habituais em saladas (24,25,26) e numa dieta saudável, especialmente entre os consumidores interessados em melhorar e manter o estado de saúde através da alteração dos hábitos alimentares. Os germinados, são apresentados hoje como excelente exemplo de alimentos com características funcionais, promotores da saúde e protetores do risco de desenvolvimento de doenças (27).

Os germinados das espécies do género *Brassica* tornaram-se particularmente populares como alimentos saudáveis, especialmente os germinados de brócolo, tendo sido recomendados na dieta humana, uma vez que, apresentam todas as vantagens associadas às sementes germinadas – elevado valor nutricional, elevado teor de compostos fitoquímicos promotores da saúde e reduzido teor de gordura (28) – sendo ainda alimentos considerados de fácil preparação, frescos e seguros, o que responde às necessidades dos consumidores preocupados com uma alimentação saudável.

2.2. Produção e alterações durante o processo produtivo

A produção de germinados corresponde à produção de plântulas com 8-10 cm de comprimento, resultante da germinação de sementes em sistemas hidropónicos, que exigem um controlo apurado das condições de germinação, nomeadamente de fatores como a temperatura e a oxigenação do ar e da água, a velocidade e caudal de recirculação da água, a humidade e a luz. Estas condições são extremamente variáveis, em função do tipo de germinado a produzir. A tecnologia associada à produção de germinados é, igualmente, muito variada, embora, a tecnologia de base para a germinação de sementes seja um processo simples e pouco dispendioso (29). Existem sistemas produtivos básicos, que requerem a simples produção em tabuleiros ou em frasco, mas há igualmente sistemas industriais com uma tecnologia mais ou menos automatizada, e com sistemas de recirculação de água, envolvendo mecanismos apurados de tratamento da água. Independentemente dos sistemas de produção utilizados existem etapas básicas que constituem o fluxo de produção de germinados (Figura 2.1).

Durante o processo de germinação das sementes verificam-se várias alterações, que variam significativamente em função da espécie, da variedade e de vários fatores associadas ao processo, nomeadamente a temperatura, a luz e o tempo de germinação (25,30,31). A germinação é um processo fisiológico complexo, tendo início com a absorção de água pela semente quiescente e terminando com a emergência do eixo embrionário (32,33). Este processo promove uma intensa atividade metabólica, envolvendo alterações estruturais subcelulares, intensa atividade respiratória, síntese macromolecular e finalmente alongamento celular, fenómenos que determinam uma melhoria nutricional das sementes (34). Vários estudos referem que durante este processo ocorre um aumento da disponibilidade de nutrientes e uma redução de fatores antinutricionais nos germinados, comparativamente com as sementes que lhes deram origem (35,36,37,38,39). No processo de germinação as reservas energéticas, armazenadas na semente, são degradadas por processos enzimáticos, produzindo novos compostos (40,41). Resultam assim proteínas de elevada qualidade, uma distribuição mais equilibrada dos aminoácidos, um elevado teor de ácidos gordos polinsaturados, um aumento da biodisponibilidade de minerais essenciais e um teor de vitaminas também mais elevado (42,43).

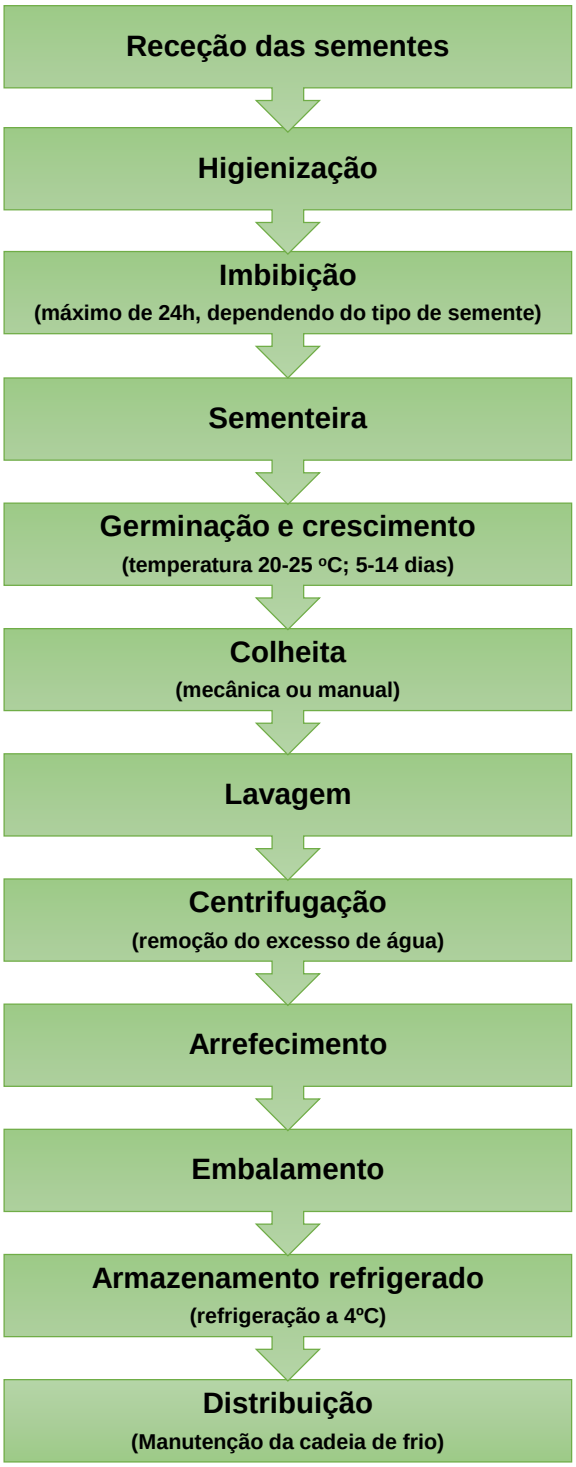

Figura 2.1 Fluxo de produção de germinados (adaptado de Vale (44) e de National Advisory Committee on Microbiological Criteria for Food, NAC-MCF (45)).

Estas alterações promovem um aumento de compostos com atividade antioxidante (46, 47), fenómeno que contribui igualmente para melhorar a qualidade nutricional dos germinados (48). Existem ainda evidências de que as sementes de *Brassica* possuem elevado teor de glucosinolatos que, após os processos metabólicos inerentes à germinação, resultam num aumento da atividade anticarcinogénica (49) e das propriedades antioxidantes (50,51,52). É este elevado teor de compostos bioativos dos germinados que está na base da sua classificação como alimentos funcionais (53).

2.3. Segurança dos germinados

O crescente consumo de germinados *prontos a consumir* gerou um aumento da incidência de surtos associados ao consumo destes produtos. Isto resulta do facto dos germinados serem um excelente veículo para o crescimento e a transmissão de agentes patogénicos (54, 55, 56, 57, 58). Se por um lado, a produção de germinados decorre a temperaturas entre 20 e 40 ^{0}C, favorável ao crescimento de microrganismos (58, 59, 60, 61,62), por outro lado, o seu elevado teor de proteínas, minerais e vitaminas (63) convertem-nos em excelentes substratos para o crescimento microbiano, incluindo microrganismos patogénicos (64).

As sementes contêm normalmente uma carga microbiana elevada, que pode variar entre 10^3 e 10^6 cfu/g, sendo esta constituída principalmente por pseudomonas, enterobactérias, bactérias láticas e fungos (65,66,67,68). Salienta-se ainda, que as sementes utilizadas para produzir germinados são produtos cuja colheita decorre em ambiente agrícola, estando expostas a múltiplas fontes de contaminação, providenciando excelentes condições para a rápida proliferação de microrganismos patogénicos (69). Nos primeiros dias de germinação a flora microbiana aumenta 2-3 unidades logarítmicas e pode atingir níveis máximos de 10^6-10^9 cfu/g, após dois dias de germinação (70). Durante o processo de germinação podem atingir-se níveis elevados de carga microbiana, da ordem dos 10^8 aos 10^{11}cfu/g (48,71). Os elevados níveis de contaminação *per se* estão na origem do reduzido tempo de vida útil dos germinados e dos elevados níveis de infeção descritos pela NACMCF (45) e por Taormina (58).

Uma ampla gama de microrganismos constitui a comunidade microbiana que normalmente ocorre nos germinados (72). Contudo, a maior parte dos surtos associados ao consumo de germinados, foi associada à presença de sementes contaminadas por enterobactérias, onde se destaca a presença de *Salmonella* e *Escherichia coli* O157:H7 e por vezes *Bacillus cereus* (45,58,59,69,73-78). No entanto, os germinados podem

igualmente ser contaminados por microrganismos patogénicos durante o processo de produção, bem como durante a colheita, embalamento e distribuição (58,64,79). O crescimento potencial de microrganismos patogénicos, tais como *Salmonella* e *E. coli* O157:H7, é particularmente preocupante neste tipo de produtos e o seu controlo constitui um desafio para a indústria de produção de germinados.

Múltiplos surtos de doenças alimentares foram associados ao consumo de germinados em cru ou parcialmente cozinhados (58,80). A população infetada pode variar desde um reduzido número de pessoas (58), a várias centenas ou até dezenas de mortes (62). A título de exemplo, destaca-se a catastrófica crise alimentar do Japão em 1996, associada ao consumo de germinados de rabanete contaminados com *Escherichia coli* O157, e que envolveu 46000 pessoas (81). Na Austrália, registaram-se, entre 2005-06, 140 casos de salmonelose associados ao consumo de germinados não cozinhados (82,83) e nos EUA o consumo de germinados resultou em 2246 casos de doenças alimentares, registadas entre 1995 e 2010 (84). Na Europa, mais concretamente na Alemanha, destaca-se o recente caso associado ao consumo de germinados de sementes de feno-grego importadas do Egito (85,86). Segundo a EFSA foram reportados 3126 casos de doenças diarreicas causadas por *Escherichia coli* (STEC), serotipo O104:H4 e, foram registadas 17 mortes não só na Alemanha mas também noutros países, incluindo a Noruega (87). Contudo a Food Safety Authority of Ireland's (FSAI) (85) aponta para mais de 47 mortes e várias centenas de infeções.

A maior parte dos consumidores não cozinha os germinados, utilizando-os como produtos *prontos a consumir*. A simples lavagem, prévia ao consumo, pode não ser eficiente para eliminar os microrganismos, particularmente os de natureza patogénica (88). A importância dada a este problema e aos surtos associados foi primeiramente refletida num conjunto de recomendações específicas desenvolvidas em 1997 nos Estados Unidos da América pela NAC-MCF (45) e também pela Food and Drugs Administration (USFDA) (89), que desenvolveu um guia de boas práticas para promover a segurança dos germinados. Este documento destaca, especificamente, cinco recomendações: (1) boas práticas agrícolas na produção de sementes para germinados, (2) boas condições higio-sanitárias de acondicionamento e armazenamento das sementes (3) boas práticas de manipulação em todas as operações associadas à produção de germinados (4) tratamentos de higienização das sementes antes de iniciar o processo de germinação (5) controlo da qualidade da água e do produto antes de entrar nos canais de comercialização (89,90-92). Na Europa, a Irlanda publicou em 2011 um guia de boas práticas para a

produção segura de germinados. No referido documento é recomendado que os germinados produzidos, segundo as regras de boas práticas publicadas, podem ser rotulados como germinados *prontos a consumir*, enquanto as produções que não obedecem a estas recomendações devem ser rotuladas como germinados que devem ser *cozinhados antes de consumir* (85).

2.4. Métodos de higienização de sementes

A contaminação com microrganismos patogénicos, de sementes e de germinados, é motivo de grande preocupação ao nível da segurança alimentar, porque, e dependendo dos níveis de contaminação, pode reduzir a qualidade das sementes e representa um risco para a saúde humana. O controlo da carga microbiana de sementes é fundamental, ainda, para minimizar os riscos de contaminação dos germinados (93), sendo recomendada a higienização das sementes antes de se iniciar o processo de germinação (48). Existem múltiplos métodos de desinfeção de sementes, contudo, nem todos são igualmente eficientes e ecologicamente "amigáveis". Um tratamento de descontaminação de sementes com elevada eficácia deve inativar a flora patogénica, preservando a viabilidade das sementes, a capacidade de germinação e o vigor das mesmas (94).

As estratégias utilizadas para minimizar os riscos associados ao aparecimento de surtos, resultantes do consumo de germinados, envolvem tratamentos prévios à germinação das sementes, com métodos de natureza química, física e combinações de ambos os tipos (45). Contudo, parece não existir um único método de tratamento de sementes capaz de eliminar eficazmente contaminações por *Salmonella* e por *E. coli* O157:H7 (54,95,96).

2.4.1. Métodos químicos

Entre as várias estratégias utilizadas para garantir a segurança dos germinados, incluem-se a utilização de desinfetantes químicos, sendo a utilização de hipoclorito uma das mais frequentemente utilizadas (77,97-100). A USFDA (89) recomenda mesmo o uso de hipoclorito de cálcio a 20000 ppm. Contudo, a indústria agroalimentar de produção de germinados apresenta algumas reservas ao uso deste produto, sobretudo por questões de segurança dos manipuladores, bem como devido ao impacto ambiental negativo associado ao produto (62). Em países como a Alemanha este tipo de tratamento não é mesmo permitido e a sua utilização não se coaduna com a designação de *germinados biológicos* (101).

Têm sido desenvolvidos trabalhos na procura de métodos alternativos e eficazes para a higienização das sementes, mas que sejam igualmente de reduzido custo, ambientalmente sustentáveis e sem impacto negativo sobre a taxa de germinação das sementes. Vários agentes químicos têm sido utilizados, incluindo o peróxido de hidrogénio, etanol, ácido lático, ácido peroxiacético, ácidos gordos (102-107) e outros desinfetantes comerciais (48). Contudo, a eficácia dos vários tipos de tratamento é muito variável e muitas vezes semelhante à do hipoclorito de cálcio 20000 ppm. Ding et al. (62) referem que a eficácia máxima, detetada nas várias linhas de investigação sobre este tema, conduziu a uma redução da carga microbiana de 7,11 log ufc/g, enquanto a maioria dos tratamentos conduz a reduções moderadas da carga microbiana, geralmente inferiores a 3,50 log ufc/g. Apesar de muitos destes métodos conseguirem atingir níveis de descontaminação microbiana de sementes, superiores a 5 log ufc/g, tal como é recomendado pela NAC-MCF (45), nenhum destes métodos consegue eliminar completamente os microrganismos patogénicos das sementes (45,102) e muitos deles resultam na diminuição da sua taxa de germinação (48). As condições utilizadas nos diferentes tipos de tratamentos químicos são muito variáveis e não existe ainda uma validação da eficácia dos mesmos, o que faz com que as recomendações da USFDA (45) continuem a ser, ainda hoje, as mais utilizadas neste tipo de agroindústria (62).

2.4.2. Métodos físicos

Para além dos tratamentos químicos existem tratamentos de natureza física, que parecem ter melhores caraterísticas de penetração, permitindo melhores resultados em situações onde os tratamentos químicos não se mostraram eficientes, como é o caso da eliminação de microrganismos patogénicos retidos em superfícies escarificadas e no interior das sementes (62). Por outro lado, os tratamentos térmicos e por altas pressões são “amigos” do ambiente e o seu uso tem vindo a ser incentivado.

Dos diferentes tipos de tratamentos físicos destacam-se os que resultam da ação do calor (108,109), da exposição a radiações ionizantes (110), de tratamentos por altas pressões (62) e de tratamentos por ultrassons (111), podendo ainda ser utilizadas combinações de tratamentos químicos e físicos (45).

Os tratamentos térmicos têm sido dos mais intensamente estudados e usados na descontaminação microbiana (112) de materiais vegetativos, incluindo de sementes, e o seu uso data já de 1920 (113). Contudo, o uso de água aquecida, para redução de patogénicos, em sementes de germinados, foi estudado pela primeira vez por Jaquette et

al. (108), tendo estes autores verificado que, tratamentos de 5 minutos com água a 57-60 ^{o}C, conduzem a uma redução de 2,5 log ufc/g na população de *Salmonella enterica*, sem que a taxa de germinação das sementes de luzerna fosse significativamente afetada. No entanto, temperaturas ligeiramente mais elevadas ou tempos de exposição mais prolongados reduzem significativamente a taxa de germinação das sementes (114). Salienta-se, ainda, que os tratamentos por ação do calor, dependendo da temperatura e do tempo de exposição, podem levar a perdas consideráveis de nutrientes, desenvolvimento de colorações indesejáveis e deterioração das propriedades organoléticas dos alimentos (115).

A aplicação de tecnologias de calor seco permite obter resultados mais interessantes (116-118). Neetoo & Chen (113) referem que o uso de calor seco (55-60 ^{0}C) *per se* é pouco eficiente na descontaminação de sementes de luzerna, contaminadas com *Salmonella*. Contudo o uso de temperaturas ligeiramente mais agressivas (65 ^{0}C) e um tempo de tratamento prolongado durante 10 dias resulta na total eliminação de *Salmonella* e de *E. coli* O157:H7, sem que a taxa de germinação das sementes seja muito afetada.

O uso de altas pressões pode ser, igualmente, uma alternativa interessante aos métodos de desinfeção químicos, uma vez que constitui uma tecnologia não térmica, capaz de reduzir a população microbiana, preservando as características dos alimentos, e mantendo ainda as suas propriedades sensoriais e nutricionais (119). O tratamento de sementes por altas pressões, com pressões entre os 500 e 600 MPa, durante 2 minutos, à temperatura ambiente, promove uma redução da carga microbiana de cerca de 3,50 log ufc/g, ou até mesmo superior (120, 121). Esta tecnologia associada a temperaturas mais elevadas pode ainda ser mais eficiente no controlo microbiano (121-123). Em média, os tratamentos por altas pressões permitem obter reduções microbianas de 5,09 log ufc/g, valores que são bem mais eficientes do que os obtidos com outros métodos, e significativamente melhores do que os obtidos com o hipoclorito de cálcio 20 000 ppm (62).

O uso de radiação ionizante tem, igualmente, mostrado uma elevada eficácia na eliminação de microrganismos patogénicos, quer em sementes quer em germinados (78). A aplicação de radiação ionizante é, atualmente, reconhecida do ponto de vista legal, em vários países, como uma técnica segura e eficiente para melhorar a segurança alimentar. A USFDA aprovou mesmo a aplicação de radiações, numa dose máxima de 1 KGy, para tratamento de produtos para consumo em fresco, como é o caso dos germinados. No caso do tratamento de sementes, para produção de germinados, a dose máxima pode atingir os 8 KGy (124). Vários estudos demonstram que a aplicação de radiação gama a germinados

de luzerna, rabanete e de feijão mungo, em doses inferiores a 2,0 KGy, reduz as populações de *Salmonella* e de *E. coli* O157:H7 para níveis não detetáveis (125,126). Thayer et al. (127) conseguiram eliminar completamente *Salmonella mbandaka* em sementes de luzerna, mediante a irradiação das sementes com radiação gama na ordem do 4,0 KGy. De forma semelhante, a aplicação de feixes de eletrões a 3,3 ou 5,3 kGy contribuiu para eliminar *Listeria monocytogenes* em germinados de luzerna (128).

A ultrasonicação é outro exemplo de tratamento físico utilizado, na última década, como alternativa ao processamento pelo calor na indústria alimentar (129). Este tipo de tecnologia tem sido igualmente utilizado na inativação de microrganismos em alimentos líquidos, mas também em frutos e vegetais (130,131). As ondas ultrassónicas de 20 a 100 kHz danificam as paredes celulares dos microrganismos, inativando-os (132-134). É uma tecnologia que reduz o tempo de processamento dos alimentos, que tem associado um reduzido consumo energético e que reduz ainda a perda de sabor dos alimentos (129). Foram ainda realizados poucos estudos sobre a aplicação desta tenologia à descontaminação de sementes e de germinados, contudo, é uma tecnologia com elevado potencial. Chiu & Sung (111) demonstraram que a ultrasonicação promove uma redução da carga microbiana da ordem dos 5,86 log ufc/g em sementes de ervilha. Este parece ser um método promissor, contudo, não existem ainda sistemas comerciais para a descontaminação de sementes à escala da produção de germinados.

2.4.3. Métodos de biocontrolo

Procuram-se, atualmente, novas estratégias de ação para o controlo microbiológico de sementes, que sejam eficientes e que não afetem nem a viabilidade nem a fisiologia das mesmas. Uma abordagem que tem sido objeto de interesse crescente é a utilização de bacteriófagos, de estirpes de bactérias e de bacteriocinas, e que têm sido testados para inibir o crescimento ou a multiplicação de microrganismos patogénicos como *Salmonella spp* e *E. coli* O157:H7, em sistemas de produção de germinados (69,135-137).

Uma das tecnologias que tem vindo a ser investigada é a utilização de bacteriófagos, enquanto agentes utilizados no biocontrolo dos alimentos (69). Os bacteriófagos são vírus que infetam unicamente bactérias e que apresentam elevada especificidade em relação ao hospedeiro (138). A utilização de bacteriófagos surge, assim, como uma alternativa aos métodos tradicionalmente utilizados na segurança alimentar e na conservação dos alimentos (139,140). Há já alguns casos de sucesso, aplicados ao controlo do crescimento de *Listeria monocytogenes*, *Salmonella spp* e *Campylobacter*

jejuni, nos setores das frutas, dos produtos lácteos e das carnes (139,141,142). Saliente-se ainda que a USFDA (143) já aprovou um bacteriófago específico para *Listeria* (Listex P100) que pode ser utilizado na conservação de alimentos *prontos a consumir*.

Esta tecnologia aplicada ao controlo de microrganismos patogénicos dos alimentos, e no caso concreto dos germinados, parece apresentar resultados satisfatórios (69). Em geral, e apesar do reduzido número de estudos realizados, os resultados obtidos sugerem que a inibição da contaminação microbiana, por estes agentes, pode atingir resultados semelhantes aos obtidos com os tratamentos à base de hipoclorito de cálcio 20000ppm (62). Contudo, há ainda muitas incertezas que impedem que esta tecnologia seja aplicada de forma generalizada. As incertezas prendem-se, sobretudo, com questões inerentes à complexidade da tecnologia de aplicação dos bacteriófagos, à atual incerteza da sua eficácia à escala industrial e a certas preocupações sobre potenciais efeitos adversos na saúde dos consumidores.

2.5. Qualidade dos germinados de *Brassica*

A qualidade alimentar deve ser entendida como um conceito complexo e multidimensional que depende, não só, das propriedades dos alimentos mas também do consumidor e da sua perceção da alimentação (144). Van Boekel (145) e Linnemann et al. (146) tendo por objetivo tornar a qualidade mais tangível no âmbito das ciências alimentares, sugerem uma distinção entre aquilo que são os atributos de qualidade intrínsecos – inerentes ao produto – e os atributos de qualidade extrínsecos – associados aos métodos de produção mas que não são propriedades próprias dos alimentos.

Os atributos de natureza intrínseca dos alimentos, e sobretudo dos de origem vegetal, providenciam estímulos aos consumidores e desempenham um papel importante na perceção da qualidade. Este tipo de atributos pode ser dividido em atributos de natureza sensorial e atributos relacionados com a saúde. Os atributos sensoriais estão relacionados com os aspetos clássicos da qualidade (sabor, aroma, aparência, cor, textura e odor) mas nas últimas décadas, os atributos relacionados com a saúde, tais como os valores nutricionais e os promotores da saúde, adquiriram uma importância igual, ou até mesmo superior, à dos sensoriais (147). As propriedades relacionadas com a presença de compostos bioativos nos alimentos de origem vegetal (glucosinolatos, compostos fenólicos e carotenoides) ajudaram na construção de uma nova imagem destes alimentos, tendo mesmo contribuído para o desenvolvimento de campanhas governamentais sobre o consumo de frutas e vegetais. Foi esta nova perceção que determinou igualmente

alterações no consumo de germinados e que, tal como já foi referido anteriormente, registou um aumento significativo nas últimas décadas, tendo influenciado igualmente o tipo de investigação realizada nesta área, e que tem sido muito focalizada em estudos de determinação do valor nutricional dos germinados (48).

Os fatores extrínsecos relacionam-se mais com questões como o uso de pesticidas, o comércio justo, o trabalho infantil, o bem-estar animal, o tipo de embalagem, o uso de determinada tecnologia de processamento, entre outros, e normalmente, não possuem influência direta nas caraterísticas dos produtos. No entanto são de extrema importância e determinantes da política de compra de alguns consumidores (146). A produção de germinados encontra aqui um campo de ação favorável, pois tem associada uma tecnologia considerada "limpa", económica e cujo principal fator de produção é a água. Desta tecnologia de produção resultam os germinados, cuja imagem, nos consumidores, está associada a alimentos frescos e do tipo biológico.

2.5.1. Qualidade nutricional dos germinados

Os principais componentes nutricionais das brassicas são as proteínas, os hidratos de carbono e as vitaminas (ácido ascórbico, ácido fólico, tocoferóis e provitamina-A). Ao nível da composição mineral destaca-se o ferro, cálcio, selénio, cobre, manganês e zinco, que são os minerais essenciais destas plantas (148).

2.5.1.1. Proteínas

A qualidade das proteínas depende do teor de aminoácidos essenciais presente, e a germinação é um processo biotecnológico durante o qual enzimas metabólicas, tais como as proteinases, são ativadas (149). Em resultado, alguns aminoácidos e péptidos podem ser degradados, sendo igualmente produzidos e utilizados outros que vão originar novas proteínas. É desta forma que a qualidade nutricional das proteínas pode ser melhorada, e é esta a principal razão, pela qual Gulewicz et al. (150) referem que a germinação é um processo tecnológico que melhora a qualidade nutricional das sementes de leguminosas e de outras espécies. Urbano et al. (151,152) conclui mesmo que há um aumento da digestibilidade das proteínas durante a germinação de sementes de ervilha.

Segundo López-Cervantes et al. (153), o teor de proteínas encontrado nos germinados é comparável ao teor proteico encontrado em alimentos considerados ricos em proteínas. Salienta-se, a título de exemplo, o teor de proteína de germinados de brócolo (22,41 g/100 g de matéria seca) que é superior ao encontrado na planta adulta (154). O aumento, durante o processo de germinação, do teor de aminoácidos, principalmente

aminoácidos essenciais, e do teor de proteína bruta foi igualmente observado por Tarasevičienė et al. (149) em germinados de brócolo. Estas alterações são o resultado de processos de hidrólise, de síntese e de rearranjos moleculares, uma vez que a germinação envolve a mobilização de reservas proteicas dos cotilédones e a síntese de novas proteínas, necessária ao crescimento dos germinados (155).

Em plantas de brócolo foram identificados 17 aminoácidos (alanina, arginina, asparagina, ácido aspártico, glicina, ácido glutâmico, glutamina, histidina, isoleucina, leucina, metionina, fenilalanina, serina, treonina, triptofano, tirosina e valina) (26,156). Nos germinados da mesma espécie foi obtido um perfil semelhante, exceto no que diz respeito a asparagina, glutamina e triptofano (149). Resultados idênticos foram obtidos também por López-Cervantes et al. (153) os quais referem igualmente, que o teor total de aminoácidos é superior nos germinados, comparativamente com as sementes de brócolo, tendo-se registado aumentos entre 3% e 42%.

Sendo o rácio entre aminoácidos essenciais e totais um dos indicadores da qualidade proteica, nos germinados de brócolo este rácio é sempre superior a 45%, e por isso mesmo, mais elevado (149) do que o encontrado na literatura (33,9%) (157), o que mais uma vez atesta a qualidade proteica dos germinados.

2.5.1.2 Teor de gordura e perfil de ácidos gordos

Existem muito poucas referências relativamente às alterações do teor de gordura e da composição em ácidos gordos que ocorrem durante o processo de germinação, especialmente em germinados de *B. oleracea*. Os principais trabalhos realizados reportam o estudo de germinados de trigo-sarraceno (158) onde os ácidos gordos insaturados predominam, destacando-se a presença do ácido linoleico (C18: 2n6).

Tokiko & Koji (159) analisaram também diferentes tipos de germinados, incluindo brócolo e couve roxa, e referem que o teor de gordura dos germinados varia entre 0,4 e 1,6%, situando-se em 0,7% e 0,8% nos germinados de brócolo e de couve roxa, respetivamente. Estes autores observaram um predomínio dos ácidos gordos insaturados (oleico C18: 1n9; linoleico, C18: 2n6 e linolénico, C18: 3n3) em ambos os tipos de germinados.

Mais recentemente, foram estudados por López-Cervantes et al. (153), germinados de brócolo e entre os ácidos gordos polinsaturados foram identificados o ácido linoleico, o linolénico e o araquidónico (C20: 4n6), destacando-se um aumento, durante o processo de

germinação, do teor dos ácidos linoleico e linolénico. Relativamente aos ácidos gordos monoinsaturados, refere-se a presença dos ácidos palmitoleico (C16: 1n7), oleico (C18: 1n9), vacénico (C18: 1n7) e gondoico (C20: 1n9) e salienta-se ainda que, segundo estes mesmos autores, o teor de ácido oleico e de ácido gondoico diminui com o período de germinação. O perfil de ácidos gordos, observado por López-Cervantes et al. (2013), é semelhante ao obtido por Zhuang et al. (160), Campas-Baypoli et al. (154) e Márton et al. (3) e, por outro lado, o aumento na proporção de ácidos gordos essenciais (C18: 2n6 e C18: 3n3), obtido nos germinados de brócolo, é semelhante ao reportado por Kim et al. (158) para o trigo-sarraceno.

O reduzido número de trabalhos realizados sobre a variação do perfil de ácidos gordos durante o processo de germinação, não permite estabelecer um padrão de variação do mesmo, contudo os estudos realizados parecem apontar para um decréscimo do teor de ácidos gordos saturados e para um aumento considerável dos ácidos gordos polinsaturados.

2.5.1.3. Composição aproximada dos germinados em hidratos de carbono, fibra e minerais

As brassicas possuem um teor de hidratos de carbono que varia ente 0,3 e 10% (numa base de peso fresco) (161), e nos germinados destas plantas, particularmente em brócolo e couve roxa, o teor deste nutriente é da ordem de, 2,0% e 2,2%, respetivamente. Contudo o teor de hidratos de carbono nos germinados e nas sementes varia consoante a espécie/variedade. López-Cervantes (153) observaram um decréscimo no teor de hidratos de carbono ao longo do processo de germinação de sementes de brócolo. Este decréscimo está associado à intensa atividade metabólica que ocorre durante o processo de germinação e que resulta da degradação das substâncias de reserva armazenadas nas sementes (162), sendo os polissacarídeos degradados em oligossacarídeos e monossacarídeos. Assim, os extratos isentos de azoto (hidratos de carbono brutos) tendem a decrescer gradualmente enquanto os açúcares redutores e o teor total de açúcares tendem a amentar, resultando num aumento da bioenergia durante o processo de germinação (163-165).

As variações no teor de fibra bruta e de fibra dietética, durante o processo de germinação das sementes, têm sido muito pouco estudadas e as principais referências encontradas são mesmo contraditórias. López-Cervantes et al. (153) observaram uma redução no teor de fibra bruta em germinados de brócolo durante o processo de

germinação. O teor mais reduzido foi observado ao fim de 11 dias de germinação (5,15g/ 100g) comparativamente ao observado nas sementes de brócolo (15,47g/100g). Contudo, estudos realizados por Tarasevičienė et al. (149) referem um aumento no teor de fibra durante 120h de germinação, e referem ainda, que as sementes de brócolo apresentam menor teor de fibra bruta do que os germinados, em qualquer dos momentos de germinação monitorizados. Estas diferenças podem estar associadas à utilização de diferentes cultivares de brócolo e também a diferenças nas condições de produção, nomeadamente no que concerne ao fator luz, pois a produção de germinados de Tarasevičienė et al. (149) decorreu em completa obscuridade, obtendo-se os germinados "brancos", enquanto López-Cervantes et al. (153) produziram os germinados em ciclos de luz e de obscuridade.

A fibra dietética corresponde a um grupo heterogéneo de substâncias quimicamente diversificadas, podendo ser classificado como fibra dietética solúvel e insolúvel, mediante o seu comportamento na presença da água. Ambas as formas possuem a capacidade de estabelecer ligações com a água e com minerais catiónicos (166), bem como com moléculas orgânicas e inorgânicas e com os sais biliares, o que influencia significativamente a absorção dos lípidos e o metabolismo do colesterol (167). A fração solúvel da fibra dietética ocorre normalmente em reduzidas proporções nos alimentos, sendo a fração insolúvel dominante em todas as plantas e também nas sementes e nos germinados (164). A fibra dietética tem elevado significado nutricional pois contribui para a manutenção da integridade do trato gastrointestinal, e estes últimos autores, analisaram a sua variação em germinados de *Brassica*. Foi observado um aumento da ordem dos 20% no teor de fibra dietética, quer solúvel quer insolúvel, durante o processo de germinação, o que indica que os germinados de brássicas podem ser considerados excelentes fontes de fibra dietética.

A presença de minerais é essencial para o normal funcionando do organismo e López-Cervantes (153) observaram um aumento na ordem dos 44% no teor de minerais, avaliado pelo teor de cinzas, em germinados de brócolo com 3 dias de crescimento. Os germinados de brássica possuem níveis mais elevados de cálcio e de magnésio, 12% e 14%, comparativamente às sementes (164), tendo sido constatado igual comportamento em relação ao cobre e zinco, que registaram, respetivamente, aumentos de 25% e de 45%. Segundo o National Research Council (168) os germinados são uma fonte particularmente rica de zinco e 100g de germinados desidratados podem providenciar 50% das RDA em

indivíduos com mais de 18 anos, o que abre uma nova perspetiva de utilização dos germinados, enquanto ingredientes, podendo ser usados em formulações alimentares.

2.5.2. Metabolitos secundários (componentes não nutricionais)

As plantas são tradicionalmente utilizadas na alimentação humana mas também na terapia de certo tipo de doenças, em resultado da presença de substâncias farmacologicamente ativas (169). Alguns autores sugerem que a valorização nutricional e medicinal das brássicas resulta principalmente do elevado teor de hidratos de carbono, proteínas, vitamina C, vitamina B2, Vitamina A e de outras substâncias bioativas, tais como glucosinolatos e flavonoides (170). O teor de compostos bioativos nestas plantas é função de vários fatores, entre os quais, o genótipo (171,172), o stress ambiental (173), as condições de crescimento (174), de armazenamento e de processamento, e ainda os métodos de cocção utilizados (175,176).

Muitos estudos têm sido desenvolvidos nos últimos cinco anos sobre a composição nutracêutica das brassicas e em particular do brócolo e de germinados de brócolo (28,172,173,177). Estudos epidemiológicos evidenciam mesmo que indivíduos que consomem regularmente elevada quantidade de vegetais da família *Brassicaceae* (brócolo) apresentam um menor risco de contrair certos tipos de cancro (178). Estas propriedades são particularmente atribuídas ao elevado teor de metabolitos secundários, tais como compostos fenólicos, flavonoides e sulforafano (glucosinolato).

2.5.2.1 Glucosinolatos

Os glucosinolatos (β-tioglucosídeos-N-hidroxisulfatados) são um grupo de compostos aleloquímicos característicos das plantas da ordem das *Capparales*, e são o único grupo de metabolitos secundários, encontrado em todas as plantas da família *Brassicaceae*, que contêm enxofre na sua composição. Todos os glucosinolatos possuem uma molécula de β-D-glucopiranose ligada a um grupo sulfato (Figura 2.2) e a uma cadeia lateral aminoacídica, cuja diversidade está associada ao tipo de aminoácidos precursores (179-181) (Figura 2.2). O grupo sulfato é normalmente equilibrado por um catião de potássio (147), sendo estes compostos armazenados nos vacúolos das plantas sob a forma de sais potássicos (182). A presença do grupo sulfato confere aos glucosinolatos propriedades fortemente acídicas e o sabor amargo/ácido característico das espécies de *Brassicacea* (183).

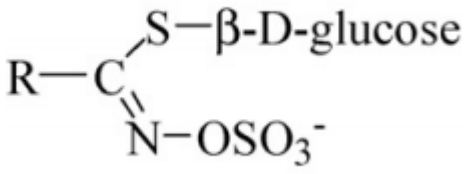

Figura 2.2 Estrutura química base dos glucosinolatos (184).

Existe uma enorme diversidade de glucosinolatos, tendo sido já isolados na natureza cerca de 120 compostos diferentes, em plantas edíveis e não edíveis, e cujas diferenças estão associadas ao tipo de cadeia lateral (180). Os glucosinolatos são divididos em três classes: os alifáticos - maior grupo presente em todas as sementes e germinados de B. *oleraceae*, *B. napus*, *B. rapa*, e *R. sativus*; os heterocíclicos – representam a menor proporção no perfil de glucosinolatos (177); e os aromáticos – característicos de *S. alba* e de *L. sativum* (28,147). Esta divisão está associada ao tipo de aminoácido precursor da cadeia lateral: nos glucosinolatos alifáticos o aminoácido precursor é a metionina, nos heterocíclicos é o triptofano e nos aromáticos é a fenilalanina (180,181,185-187). Na tabela 2.1 são apresentados os glucosinolatos normalmente encontrados nas espécies de *Brassicaceae*.

Tabela 2.1 Nomes comuns e nomenclatura dos Glucosinolatos (Gls) usualmente encontrados na família *Brassicaceae* (147)

Designação comum	**Nomenclatura química da cadeia lateral**
Gls Alifáticos	
Sinigrina	2-Propenil
Gluconapina	3-Butenil
Glucobrassicanapina	4-Pentenil
Progoitrina	2(R)-2-hidroxi-3-butenil
Epiprogoitrina	2(S)-2Hidroxi-3-butenil
Gluconapoleiferina	2-Hidroxi-4-pentenil
Glucoibervirina	3-Metiltiopropil
Glucoerucina	4-Metiltiobutil
Desidroerucina	4-Metiltio-3-butenil
Glucoiberina	3-Metilsulfinilbutil
Glucorafanina	4-Metilsulfinilbutil
Glucorafenina	4-Metilsulfinil-3-butenil
Glucoalissina	5-Metilsulfinilpentenil
Glucoerisolina	3-Metilsulfonilbutil
Gls Indólicos	

Designação comum	Nomenclatura química da cadeia lateral
Glucobrassicina	3-Indolilmetil
4-Hidroxiglucobrassicina	4-Hidroxi-3-indolilmetil
4-Metoxiglucobrassicina	4-Metoxi-3-indolilmetil
Neoglucobrassicina	1-Metoxi-3-indolilmetil
Gls Aromáticos	
Glucotropaeolina	Benzil
Gluconasturcina	2-feniletil

Diferentes espécies de brássicas e diferentes cultivares da mesma espécie apresentam teores de glucosinolatos muito variáveis (180,188,189). Numa mesma planta, apesar de existirem glucosinolatos em todos os órgãos, constatam-se diferenças, quer no perfil, quer na concentração destes compostos, o mesmo se verificando em diferentes fases do desenvolvimento das plantas (147). A título de exemplo salienta-se o caso dos germinados de rabanete que possuem nos cotilédones uma concentração de glucosinolatos cinco vezes superior à das raízes, enquanto na planta adulta estes compostos estão principalmente concentrados na raíz (190).

Os glucosinolatos coexistem nas plantas com isoenzimas do tipo mirosinases (glucohidrolase β-tioglucosideo; E.C. 3.2.1.147) (191,192) mas encontram-se armazenados em locais diferentes. Quando se verificam danos celulares, resultantes de ações de processamento, de operações de corte dos vegetais ou da mastigação, os glucosinolatos ficam em contacto com as enzimas e, na presença da água, transformam-se em compostos biologicamente ativos (193,194). Os produtos resultantes da hidrólise apresentam diferentes estruturas e diferentes propriedades físico-químicas, dependendo do tipo de glucosinolato que lhes dá origem e das condições em que decorre o processo de degradação (195), nomeadamente da presença de iões metálicos e de proteínas específicas (196). Entre os produtos de hidrólise incluem-se isotiocianatos (muitas vezes referidos como óleos de mostarda e responsáveis pela pungência das brássicas), nitrilos, epitionitrilos, oxazolidina-2-tionas e tiocianatos (197,198) (Figura 2.3).

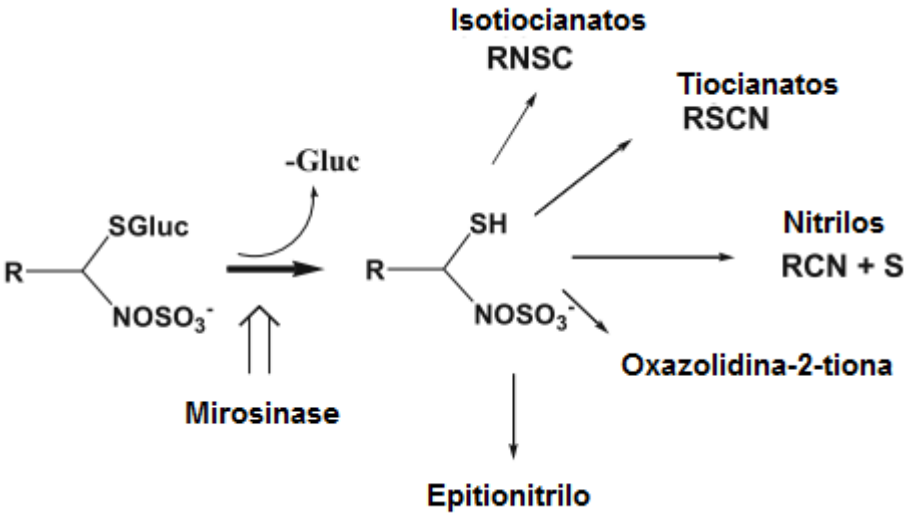

Figura 2.3 Formação de metabolitos derivados dos glucosinolatos (199).

O efeito anticancerígeno atribuído às brássicas tem sido diretamente relacionado com o teor de glucosinolatos presente nestas plantas. Vários estudos realizados *in vitro* e *in vivo* referem que os isotiocianatos possuem um papel ativo nas diferentes etapas do desenvolvimento de neoplasias, incluindo a ação na modulação das enzimas da fase I e da fase II. Os isotiocianatos funcionam como antioxidantes diretos ou indiretos, através da inibição das enzimas da fase I, da indução das enzimas da fase II, da modulação da sinalização celular, da indução da apoptose, do controlo do ciclo celular e da redução das infeções por *Helicobacter* (200).

Desde os primeiros estudos, que revelaram o potencial anticancerígeno dos glucosinolatos, que o isotiocianato sulforafano, resultante da glucorafanina, continua a ser considerado o mais potente indutor das enzimas da fase II (201). Existem ainda outros isotiocianatos, tais como a iberina (resultante da glucoiberina), o feniletil-isotiocianato (resultante da gluconasturcina) e o prop-2-enil-isotiocianato (resultante da sinigrina) aos quais são igualmente atribuídas caraterísticas indutoras das enzimas da fase II e atividade anticancerígena (202-205).

A concentração de glucosinolatos potencialmente benéficos para a saúde é superior nos germinados do que nas plantas em pleno estado de maturação. A germinação de sementes permite a disponibilização de elevadas quantidades de glucosinolatos, comparativamente às plantas adultas, e particularmente no brócolo existem diferenças

significativas, entre os germinados e as inflorescências. Os germinados de brócolo contêm teores de glucosinolatos vinte vezes superiores aos da planta adulta (153). Este tipo de germinados é particularmente rico em sulforafano, um composto com elevado potencial anticancerígeno (206), e que possui ainda atividade antimicrobiana contra *Helicobacter pylori* (207). Esta é a principal razão, pela qual, os estudos realizados sobre a composição nutracêutica de brócolo e de germinados de brócolo aumentaram significativamente nos últimos cinco anos (28,153,172,173,177,208).

Fahey et al. (50) referem que os germinados de *Brassica* possuem uma atividade protetora contra a carcinogénese, e que pode ser entre 10 a 100 vezes superior à obtida com as plantas adultas, pelo que a introdução de reduzidas quantidades de germinados na dieta permite obter um elevado efeito anticancerígeno. Contudo, os estudos realizados sobre o teor de glucosinolatos em germinados são ainda reduzidos e a maior parte deles está focalizada no estudo de germinados de brócolo, sendo importante avaliar o potencial inerente a outras espécies de *Brassica*, bem como a outras cultivares de *B. oleracea*.

2.5.2.2 Compostos fenólicos

Os efeitos benéficos das brássicas não se esgotam nas potencialidades dos glucosinolatos, enquanto compostos com propriedades anticancerígenas, mas estão também relacionados com a mistura complexa de compostos fitoquímicos que possuem atividade antioxidante, na qual se destacam os compostos fenólicos. Estes, são compostos resultantes do metabolismo secundário e muitas vezes sintetizados pelas plantas em resposta a situações de stress (7,209,210). Tal como os glucosinolatos, os compostos fenólicos possuem propriedades bioativas com interferência na saúde humana (210) e, juntamente com o ácido ascórbico, constituem mesmo o maior grupo de antioxidantes das *Brassicaceae*. Comparando a sua ação com a dos antioxidantes solúveis em lípidos, pode afirmar-se que estes últimos são responsáveis, por apenas, 20% da capacidade antirradicalar destas plantas (8). O potencial antioxidante destes compostos resulta das suas propriedades químicas, enquanto doadores de hidrogénio, atuando assim como potenciais sequestradores de radicais livres (211).

O termo compostos fenólicos corresponde a um grupo de substâncias, que inclui mais de 8000 estruturas diferentes (12), as quais são categorizadas em classes, dependendo da sua estrutura química, e subcategorizadas de acordo com o número e a posição do grupo hidroxilo e a presença de outros constituintes (210). Estes compostos podem ocorrer na forma livre ou ligados a açúcares (glicosídeos) e proteínas (212),

podendo ainda variar desde compostos de reduzida massa molecular, com um único anel aromático, até taninos e polifenóis complexos (213,214).

A classificação destes compostos é feita com base no número e no arranjo dos átomos de carbono, sendo classificados em compostos flavonoides (flavanóis, flavonóis, flavanonas, flavonas e antocianinas) e compostos não flavonoides (ácidos benzóicos, ácidos cinâmicos, estilbenos e isoflavonas) (12). Nas brássicas os compostos fenólicos mais abundantes são os flavonoides, principalmente os flavonóis e as antocianinas, e os ácidos hidroxicinâmicos (11).

Os flavonoides constituem o grupo de compostos fenólicos mais amplamente disperso e mais diversificado. São compostos formados a partir dos aminoácidos aromáticos fenilalanina e tirosina, são derivados da benzo-y-pirona e constituídos a partir de um esqueleto de flavona (C_6-C_3-C_6). A estrutura base dos flavonoides (Figura 2.4) é constituída por dois anéis benzénicos (A e B) e por um terceiro anel (C), que pode ser um pirano heterocíclico (Figura 2.4a) (flavanóis e antocianinas) ou uma pirona (Figura 2.4 b) (flavonóis, flavonas, isoflavonas e flavanonas).

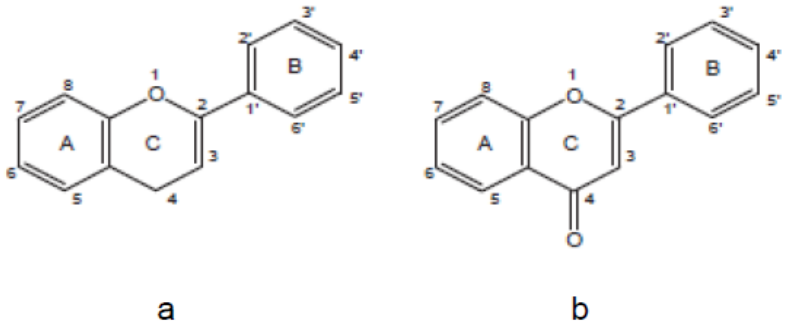

Figura 2.4 Estrutura básica dos flavonoides; a - flavonoides com anel pirano heterocíclico e b - Flavonoides com anel pirona (Adaptado de Huber (215)).

A estrutura básica destes compostos pode sofrer alterações do tipo glicosilação, esterificação, amidização e hidroxilação, entre outras, o que determina diferenças na sua atividade biológica (216).

Até ao momento foram identificados mais de 4000 compostos diferentes de flavonoides, muitos dos quais são responsáveis pelas cores atrativas das flores, dos frutos e das folhas, particularmente pelas cores amarelo, laranja e vermelho (217,218).

Os flavonóis são o grupo mais abundante e disperso dos flavonoides, onde se destacam os compostos Campferol, Quercetina e Isoramnetina, como os mais abundantes nas plantas do tipo brássicas, e que ocorrem normalmente na forma glicosilada (12).

Nas brássicas destaca-se ainda o grupo das antocianinas, que na sua forma não glicosilada são designadas por antocianidinas (agliconas). A diversidade estrutural destes compostos está associada ao número e posição dos grupos hidroxilo e metóxido, nos anéis aromáticos, e determina alterações na cor das antocianidinas. As antocianinas mais comuns são a pelargonidina, cianidina, delfinidina, peonidina, petunidina e a malvidina, sendo a cianidina o grupo mais comum nas plantas do género *Brassica* (219,220).

Entre os compostos fenólicos não flavonoides destaca-se o grupo dos ácidos fenólicos e mais concretamente os ácidos hidroxicinâmicos, compostos aromáticos com três carbonos que formam uma cadeia lateral (C6-C3) e cujos exemplos são os ácidos p-cumárico, cafeico, ferúlico e sinápico.

Vários estudos revelam a presença dos compostos fenólicos em diferentes plantas do género *Brassica* (221-227). Entre estas plantas, a cultivar brócolo, tem sido a mais intensamente estudada, e os vários estudos realizados revelam um elevado potencial antioxidante, associado ao elevado teor de compostos fenólicos (200,228,229). Segundo Heimler et al. (230) o brócolo juntamente com as cultivares que representam as plantas vulgarmente conhecidas como couves, são as que apresentam maior teor de compostos fenólicos e de flavonoides.

Contudo, o perfil fenólico dos germinados é diferente do perfil das plantas adultas. Os germinados, devido ao seu estádio fisiológico, apresentam um perfil fenólico maioritariamente composto por ácido sinápico e derivados (12). Tem uma reduzida proporção de flavonoides, principalmente quercetina e campferol na forma glicolisada (O-glicósidos), bem como a isorametina, que é caraterística de *B. rapa*, e outros ácidos hidroxicinâmicos (cloragénicos, *p*-cumárico e ferúlico) (225,231). Esta composição dos germinados vai influenciar o potencial antioxidante dos mesmos.

O ácido sinápico apresenta um elevado potencial de sequestro de peroxinitritos, tendo assim um importante papel na defesa celular, uma vez que, a sua presença, pode minimizar as alterações celulares mediadas por peroxinitritos (232). Constata-se que a germinação aumenta os níveis de ácidos fenólicos, bem como a atividade antioxidante dos germinados, pelo que estes alimentos são considerados uma importante fonte de antioxidantes naturais e Paja et al. (233) referem mesmo que os germinados liofilizados

apresentam potencial para utilização como ingredientes de alimentos funcionais na indústria alimentar.

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CAPÍTULO 3

Effect of sprouting and light cycle on antioxidant activity of *Brassica oleracea* varieties

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Effect of sprouting and light cycle on antioxidant activity of *Brassica oleracea* varieties

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ABSTRACT

The antioxidant activity of sprouts from four *Brassica oleracea* varieties was evaluated using "*in vitro*" methods (total phenolic and flavonoid content; radical scavenging assays: DPPH•, hydroxyl and peroxyl; and Ferrous Ion-chelating Ability Assay). Light cycles and sprouting influenced the potential antioxidant activity of sprouts and significant differences were observed between varieties. Generally, antioxidant activity decreased with sprouting and increased in the presence of light, whose discriminant effect was highly significant ($P < 0.001$). Red cabbage sprouts produced under light cycles showed the highest antioxidant activity (57.11 μg.mL^{-1} Ferrous Ion-chelating Ability, 221.46 μg.mL^{-1}

Hydroxyl radical scavenging, 279.02 μg.mL^{-1} Peroxyl radical scavenging). Among the traditional Portuguese brassica varieties, Penca cabbage sprouts produced under light presented higher antioxidant capacity, and also higher phenolic and flavonoid content (54.04 mg GAE.g^{-1}d.w. extract and 21.33 QE.g^{-1} d.w. extract, respectively) than Galega kale. The phenolic content of *Brassica* sprouts had a significant contribution to the antioxidant capacity.

Keywords: *Brassica* sprouts; sprouting; light cycle; antioxidant activity.

3.1. Introduction

The association between the consumption of vegetables and the reduction of chronic diseases, including cardiovascular diseases and cancer has been recognized for a long time (Bjorkman, et al., 2011; Szajdek & Borowska, 2008). Of particular importance in the prevention of these pathologies are the plants belonging to the *Brassica* genus (Li et al., 2010; Podsędek, 2007) as a result of its high levels of vitamins, minerals, phenolic compounds and glucosinolates (Moreno, Carvajal, Lopez-Berenguer, & Garcia-Viguera, 2006). Moreover, phytochemicals like ascorbic acid and phenolic compounds have high antioxidant activity, contributing in a significant way to the beneficial effects of the brassica plants intake (Castañeda-Ovando, Pacheco-Hernández, Páez-Hernández, Rodríguez, & Galán-Vidal, 2009; Pérez-Balibrea, Moreno, & García-Viguera, 2008).

Germinated seeds have a higher nutritious potential than the raw ones, with a simple, fast and economic production (Martinez-Villaluenga et al., 2010). Besides that, the antioxidant and anticancer potential of brassica plants has contributed to the added value of its edible sprouts, not only for consumers worried with a healthy diet, but also for scientific community (Martinez-Villaluenga, et al., 2010; Moreno, Pérez-Balibrea, Ferreres, Gil-Izquierdo, & García-Viguera, 2010; Zielinski, Piskula, Michalska, & Kozlowska, 2007). Some of the health-promoting factors may be present ten times higher in sprouts than in

mature vegetables (Martinez-Villaluenga, Frias, Gulewicz, Gulewicz, & Vidal-Valverde, 2008; Yuan, Wang, Guo, & Wang, 2010). This is the case of the flavonoids content and other phenolic compounds that clearly contribute to the antioxidant potential (Galati & O'Brien, 2004). Among the most studied Brassica sprouts the broccoli outstands for its properties in the oxidative stress reduction, its potential anticarcinogenic activity (Fahey, Zhang, & Talalay, 1997) and its high concentration of phenolic bioactive compounds (Pérez-Balibrea, Moreno, & García-Viguera, 2008). However, it is known that genetic and environmental factors (Aires et al., 2011; Martinez-Villaluenga, Frias, Gulewic, Gulewicz &Vidal-Valverde, 2008) among others (Podsędek, 2007) affect the yield and accumulation of bioactive compounds, being of utmost importance to optimize sprouts development in order to enhance their biological potential (Moreno et al., 2010). Additionally, it seems of great importance to study the behavior of other Brassica sprouts, such as red cabbage (*Brassica oleracea* var. capitata f. rubra) and varieties which consumption in Portugal is deeply rooted and associated to the traditional gastronomy, namely the Portuguese Galega kale (*B. oleracea* var. acephala DC) and Portuguese Tronchuda cabbage (*B. oleracea* L. var. costata DC landrace Penca da Póvoa), for which the studies on sprout antioxidant properties are scarce. Portuguese are one of the greatest brassica consumers in the entire world and since the beneficial effects of *Brassica* plants have been partly attributed to the compounds which possess antioxidant activity it is important to analyze the behavior of sprouts from *Brassica* varieties which represent high food consumption.

The main purpose of this work was to evaluate the antioxidant potential of aqueous extracts of sprouts of these four *Brassicaceae* varieties. In order to study the influence of the germination time and photoperiod conditions on the antioxidant profile of these varieties, different germination times and photoperiod conditions were used to produce green sprouts (GS) (cycles of light and darkness) and white sprouts (WS) (total darkness). The antioxidant capacity of the sprouts was evaluated using different “in vitro” assays, like DPPH, hydroxyl

and peroxyl radical scavenging assays. Additionally ferrous iron-chelating ability, total phenolics and total flavonoid contents were also determined.

3.2. Materials and Methods

All chemicals, reagents and solvents were analytical grade purchased from Sigma Chemical Co. (St. Louis, MO, USA). The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA, USA).

3.2.1. Plant material

Seeds from four *B. oleracea* varieties, Broccoli (*B. oleracea* L. var. italica Plenck, cultivar calabrese), Portuguese Galega kale (*B. oleracea* var. acephala DC), Portuguese Tronchuda cabbage (*B. oleracea* L. var. costata DC, landrace Penca da Póvoa) and red cabbage (*B. oleracea* var. capitata f. rubra) were acquired and germinated to obtain sprouts. Untreated seeds were acquired through the Germisem- Sementes Lda (broccoli and red cabbage seeds) and directly from the producers in Póvoa do Varzim, North of Portugal (Portuguese Tronchuda cabbage and Portuguese Galega kale).

3.2.2 Sprouting method

The sprouting method was based on the procedure described by Martinez-Villaluenga, et al. (2010) with slight adjustments. The untreated seeds were previously cleaned with a sodium hypochlorite solution (0.07 %, v/v) for 30 min, drained and washed with distilled water until they reached a neutral pH. Afterwards they were soaked in water for 12 h in darkness, at room temperature and light agitation.

The seedbed was polypropylene trays (10×15×4 cm) with inert substrate of vermiculite. The sprouts production occurred in a plant growth chamber (Fitoclima 200), at controlled temperature (25 ºC) and different photoperiod regimes. For green sprouts (GS) production a cycle of 16 h of light and 8 h of darkness was used. In the case of white sprouts (WS) the germination occurred at darkness. Germination process was carried out in

triplicate for each germination stage, with a 98 % yield rate. Germination time was different for GS and WS, as a result of differences in sprouts growth. GS were harvested after 7, 9, 12 and 15 days of germination and WS after 5, 6, 7, 9 and 12 days, keeping three common harvest dates.

After harvest the sprouts were immediately frozen at -80 ºC and lyophilized in a Telstar Cryodos-80 (Terrassa, Barcelona). The lyophilized sample was triturated to a fine powder in a knife mill (GM 200, RETSCH, Haan, Germany) and stored protected from light, oxygen and heat until analysis.

3.2.3 Preparation of aqueous extracts from the sprouts

A 0.5 g of freeze-dried samples were extracted twice with distilled water (final volume 50 mL), during 1 h, under stirring and light protection. Then the extraction followed in an ultrasonic bath at room temperature for 20 min. Finally the extracts were filtered (Whatman No. 1 paper), frozen at -80 ºC and lyophilized. The freeze-dried extracts were kept in desiccators, in the dark.

3.2.4 Total phenolic assay

The total phenolic content (TPC) of the extracts was determined according to Javanmardi, Stushnoff, Locke, and Vivanco (2003) with minor modifications. The Folin-Ciocalteu reagent was used with gallic acid as positive control. The extracts were dissolved in milliQ purified water (10 $mg.mL^{-1}$) and 50 µL aliquots or deionized water (control) were mixed with 2.5 mL 1/10 dilution of Folin-Ciocalteau reagent in a 10 mL screw-cap tube. After adding 2 mL of Na_2CO_3 (7.5%, w/v) the tube was closed and kept at 45 ºC for 15 min. The absorbance of all samples (triplicates) was measured at 765 nm using a UV–Vis spectrophotometer (Shimadzu UV-16A, Shimadzu, Corporation, Kyoto, Japan). The TPC was calculated using a calibration curve traced with gallic acid (GA, n = 3) [absorbance at 765 nm = 1.1978 CGA ($\mu g \cdot mL^{-1}$) – 0.024, R^2 = 0.9991)] and expressed as mg of gallic acid equivalent per g of dried extract (mg GAE g^{-1}).

3.2.5 Total flavonoid content

The total flavonoids content (TFC) was determined with aluminum chloride ($AlCl_3$) according to Zhishen, Mengcheng, and Jianming (1999) using quercetin as positive control. Briefly, 100 µL of the extract (10 $mg.mL^{-1}$) were added to 300 µL of distilled water followed by 30 µL of $NaNO_2$ (5%). After 5 min at 25 ºC, 30 µL of $AlCl_3$ (10%) were added and the solution allowed to stand for more 5 min. Then, the reaction mixture was treated with 200 µL of NaOH (1 mM) and completed the volume to 1 mL with distilled water. The absorbance of the mixture was then determined at 510 nm against a water blank. All tests were performed in triplicate and the flavonoids content was calculated from a quercetin standard curve [absorvance at 510 nm = 0.0004 $C_{quercetin}$ ($\mu g \cdot mL^{-1}$) + 0.0044, R^2 = 0.9993)]. Results were expressed as quercetin equivalents (mg quercetin g dried extract $^{-1}$).

3.2.6 DPPH scavenging activity

The DPPH scavenging activity was determined according to a method previously described (Fukumoto & Mazza, 2000) with some modifications. The sample extracts activity was determined spectrophotometrically, in a Microplate Reader (BioTek Synergy HT) by monitoring the disappearance of DPPH at 515 nm. For each extract, a dilution series (seven different concentrations) was prepared with milliQ purified water in a 96 well plate, starting with 2 $mg.mL^{-1}$ of extract concentration. Each concentration (100 µL extract) was mixed with 100 µL of a solution of DPPH (150 µM prepared in 96% ethanol). The mixture was vigorously shaken and left to stand for 20 min in the dark (until stable absorbance values were obtained). Controls containing milliQ purified water instead of extract solution and blanks containing 96 % ethanol instead of DPPH solution were also made. The scavenging activity (%) was calculated according to the following formula: DPPH scavenging activity (%) = [1 - (Abs. of sample – Abs. of blank)/ (Abs. of control)] x 100. The percentage of scavenging activity was plotted against the sample concentration to obtain IC_{50}, defined as

the concentration of sample necessary to cause 50% inhibition. The experiments were performed in triplicate and ascorbic acid was used as positive control.

3.2.7 Hydroxyl radical scavenging

The hydroxyl radical scavenging activity was determined according to a method previously described (Paya, Halliwell, & Hoult, 1992). Hydroxyl radical was generated in a Fe^{3+}-ascorbate-EDTA-H_2O_2 system by incubation for 60 min at 37 °C a reaction mixture of $FeCl_3$ (20 µM), ascorbic acid (50 µM), H_2O_2 (1.42 mM), 2-deoxy-2-ribose (2.8 mM), EDTA (100 µM) and several concentrations (100-800 µg/mL) of tested extracts in 1 mL KH_2PO_4-KOH buffer (10 mM, pH 7.4). After incubation, 2-deoxy-2-ribose damage was measured using the thiobarbituric acid test, i. e., 1 mL of trichloroacetic acid (2.8%, w/v) and 1 mL of thiobarbituric acid (1%, w/v) were added to the reaction mixture and were incubated at 100 ºC for 15 min to develop color due to the malondialdehyde-like product of deoxyribose damage. After cooling, the absorbance was measured in an UV-Vis spectrophotometer (UV1800 Shimadzu, Kyoto, Japan) at 532 nm. Scavenging activity was expressed as the percentage inhibition of the deoxyribose degradation (% inhibition = $[(A_0-A_1)/A_0)*100]$). Additionally, the percentage of scavenging activity was plotted against the sample concentration to obtain IC_{50}, This assay was also performed without ascorbic acid to evaluate a possible pro-oxidant effect. All tests were performed in triplicate and mannitol was used as positive control.

3.2.8 Peroxyl radical-scavenger activity

Peroxyl radicals ($ROO^{\bullet}$) were generated by thermal decomposition of an aqueous-soluble azocompound, 2,2'-azobis (2-amidinopropane) dihydrochloride (ABAP, also AAPH in the literature). The $ROO^{\bullet}$ scavenging activity was assessed by monitoring the decay in turbidity, at 450 nm, of *Micrococcus lysodeikticus* suspensions, due to the inhibition of lysozyme by peroxyl radicals (Payá et al. 1992). Several concentrations of extracts were incubated with lysozyme (68 µM), ABAP (10 mM) and KH_2PO_4-KOH (50 µM, pH 7.4) to a

final volume of 1 mL, for 90 min at 45 ºC. The reaction mixture was cooled in ice and 50 µL were added to 950 µL of *M. lysodeikticus* suspension (0.3 $mg.mL^{-1}$) dissolved in Dulbecco's buffer. The decay in turbidity was measured at 450 nm, at 0.5 s intervals, during 1 min, and the average change in absorbance per minute (dA/min) was determined. Controls containing milliQ purified water instead of extract solution and blanks containing KH_2PO_4-KOH buffer (50 µM, pH 7.4) instead of ABAP solution were also made. No direct effect was observed between the tested samples and lysozyme activity. The scavenging activity (%) was calculated according to the following formula: peroxyl scavenging activity (%) = [1 - (dA/min of blank – dA/min of sample)/ (da/min blank- dA/min control)] x 100. The results were expressed as IC_{50}, representing the extract concentration required to capture 50% of peroxyl radicals, i.e. the concentration required to inhibit 50% of lysozyme activity. All tests were performed in triplicate and Mannitol was used as positive control.

3.2.9 Ferrous Ion-chelating Ability Assay

The ability of the sample extracts to chelate ferrous ions (Fe^{2+}) was evaluated by the method described by Dinis, Maderia, and Almeida (1994) with modifications. The method was tested and developed in a 96 well plate and the absorbance measured at 562 nm in a Microplate Reader (BioTek Synergy HT). 10 µL of $FeCl_2$ (50 µM) were added to 50 µL of sample extracts in different concentrations (from 25 to 250 $\mu g.mL^{-1}$) and 120 µL of methanol. The mixture was allowed to stand for 5 min and the reaction was initiated by addition of 20 µL of 3-(2-pyridyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4-triazine (ferrozine, 100 µM). The mixture was shaken and left to stand at room temperature for 10 min. After, the absorbance was measured. Controls containing milliQ purified water instead of extract were also made.

The inhibition (%) of the complex formation ferrozine–Fe^{+2} was calculated as [1 - (Abs of sample/ Abs of control)] x 100. The results were expressed as IC_{50} values ($\mu g.mL^{-1}$), representing the extract concentration required to inhibit in 50% the formation of

the complex ferrozine-Fe^{2+}. All tests were performed in triplicate and EDTA was used as positive control.

3.2.10 Statistical analyses

Data were reported as means ± standard error (SE) of at least triplicate experiments. Statistical analysis of the results was performed with SPSS 19.0 (SPSS Inc., Chicago, IL, USA). Two-way analysis of variance (ANOVA), multiple comparisons and planned means comparisons were carried out to test for any significant differences between the means. Differences at the 5% confidence level were considered significant. Correlation coefficients (r) to determine the relationship between variables were calculated using the Bivariate correlation statistical function. Also, cluster and linear discriminant analysis (LDA) were performed in order to understand the behavior of the studied factors.

3.3. Results and Discussion

Brassica plants have high antioxidant potential comparatively to other vegetable crops, particularly Broccoli and kale, which have the highest potential (Ou, Huang, Hampsch-Woodill, Flanagan, & Deemer, 2002). Sousa et al. (2008) described the antioxidant capacity against the DPPH radical and several reactive oxygen species (ROS) (superoxide radical, hydroxyl radical and hypochlorous acid) of Portuguese cabbages, but no studies were made with the sprouts of these varieties. The same situation occurs with the red cabbage, rich in colored flavonoids that are considered as multifunctional components with antioxidant activity and other beneficial biological properties (Moreno et al., 2010).

Despite the several data published by different research teams its comparison and interpretation is difficult because the natural compounds can present different ways of action. Each test performed *in vitro* only reflects the chemical activity of the compounds under defined specific conditions (Huang, Ou, & Prior, 2005) and this is why the evaluation

of the antioxidant activity should involve different methods. In this paper the DPPH, hydroxyl and peroxyl scavenging activity as well as the iron chelating effect of four varieties of *Brassicaceae* is described. Additionally the total phenolic compounds (TPC) and total flavonoid content (TFC) was outlined and correlations between the results obtained in all these assays were established. Moreover the effect of germination time and sprouting conditions is also discussed.

The germination time for each assay was based on preliminary studies. Short germination periods yielded sprouts not sufficiently developed, and long periods resulted in overgrowth and yield loss. The harvest took place when the sprouts had a size similar to the commercial one and harvesting days were different in sprouts produced under light or dark condition since darkness stimulates a faster growth, and after 15 days of germination it was verified a significant yield decrease ($p<0.05$).

3.3.1 Total phenolic compounds (TPC)

Phenolic compounds are ubiquitous phytochemicals in plants, considered to be potent antioxidants and exhibiting a wide range of physiological properties. The amounts of TPC determined in the aqueous extracts of sprouts produced in photoperiod (GS) and in darkness (WS) is presented in Table 3.1. Germination time and variety are responsible for significant differences ($P < 0.05$) on its contents. In general, WS seems to have higher TPC contents in all varieties, except in red cabbage. The high levels of anthocyanins, the flavonoids responsible for the characteristic color of this variety, could influence the statistically different behavior described once anthocyanins are recognized for possess strong free radical-scavenging properties (Renis et al., 2008). Only Penca cabbage sprouts presented similar TPC values in both methods of sprouting on the 7th day of germination. In the 9th day this situation occurred with Penca cabbage and Galega kale but only with Galega kale in 12th day. Red cabbage and Penca cabbage WS behaved similarly in what concerns its TPC contents. Those values decreased about 10% along all the experiment (from day 5

to day 12). In WS of Galega kale and broccoli the TPC increased from day 5 to day 7, followed by a decrease in the other days of the experiment (day 9 to day 12) of 20% and 10%, respectively. In what concerns the sprouting with photoperiod (GS) only broccoli and red cabbage presented increasing values till 12th day, decreasing in the 15th day. Galega kale TPC increased till 9th day and Penca cabbage showed the maximum value at 7th day, decreasing till 15th day.

3.3.2. Total flavonoid content (TFC)

The TFC results obtained in the different sprouting conditions of the studied samples are showed in Figure 3.1. For all studied *Brassicaceae* varieties the TFC seems to be affected by light, as GS are always richer in TFC than WS. Broccoli is the variety less influenced by light, with an increase in the GS of only 13.1%. The influence of light was more effective in red cabbage, with GS presenting an increase of 49% in relation to the WS. This effect could be related to higher anthocyanin content in red cabbage's sprouts produced in GS. In fact, an increase of anthocyanin contents in sprouts submitted to the effect of light has already been described (Paśko et al., 2009). In general, most plants grown in darkness accumulate less anthocyanin comparatively to light grown plants, being this effect controlled by multiple regulatory genes and induced by several factors like light (Taylor & Briggs, 1990). The presence of anthocyanins in red cabbage sprouts gives an added value to these products, due to the great importance of these pigments as a multifunctional component of foods, namely on the antioxidant activity and other beneficial biological properties (Moreno at al., 2010). The accumulation of TFC in Brassica sprouts is also significantly influenced ($p<0.05$) by the time of germination and the variety (Figure 3.1).

It seems important to point out that the day 15 of germination corresponds always to the lowest TFC in all varieties. Concerning the highest TFC, the studied varieties behaved differently. Broccoli GS showed the highest content after 12 days of germination (24.0 mg QE.g^{-1} d.w. extract), red cabbage and Penca cabbage after 7 days (41.2 mg and 24.7 mg

QE.g^{-1} d.w. extract) and Galega kale after 9 days of germination (25.4 mg QE.g^{-}1 d.w. extract). Comparing TFC between sprouts and mature plants, it was verified that GS from broccoli have higher contents than the ones found by Aires et al. (2011) in the mature plant in summer-winter season production and in a two years study. Penca cabbage GS produced similar or higher TFC than the described by the same authors as well as red cabbage GS after 7 days of germination. These results confirm the higher content of flavonoids in seed sprouts of *Brassicaceae*.

Table 3.1 Total phenolic compounds expressed as gallic acid equivalent (mg GAE.g-1d.w. extract).

Brassica variety	Germination time (days)											
	5		6		7		9		12		15	
	GS	WS	GS	WS	GS	WS	GS	WS	GS	WS	GS	WS
Red cabbage	nq	59.02±0.88^{g,h}	nq	58.32±0.30^{g,h}	72.25±0.46^{*b}	57.12±0.23^{g}	73.77±0.32^{*c}	55.33±0.12^{f}	74.97±0.044^{*d}	52.81±0.53^{e}	59.50±0.044^{a}	nq
Galega kale	nq	56.55±0.34^{g}	nq	58.53±0.20^{h}	53.59±0.89^{*c}	62.60±0.35^{i}	54.83±0.25^{c}	54.73±0.19^{f}	50.35±0.49^{b}	51.05±0.46^{e}	43.86±0.24^{a}	nq
Penca cabbage	nq	61.09±0.23^{h}	nq	60.58±0.58^{h}	61.34±1.19^{c}	58.76±0.76^{g}	53.90±0.51^{b}	55.47±0.38^{f}	50.80±1.03^{*a}	53.97±0.27^{e}	50.10±0.68^{a}	nq
Broccoli	nq	61.57±0.69^{f}	nq	62.73±0.19^{g}	54.12±0.23^{*b}	63.16±0.16^{g}	54.26±0.34^{*b}	61.05±0.18^{f}	57.20±0.30^{*c}	59.94±0.44^{e}	48.34±1.07^{d}	nq

GS means not sharing a common letter, between a and d, in a line are significantly different at p<0.05.

WS means not sharing a common letter, between e and i, in a line are significantly different at p<0.05.

***** Means significant differences (p<0.05) between GS and WS at the same germination time.

nq - have not been quantified because sprouts yields was very low

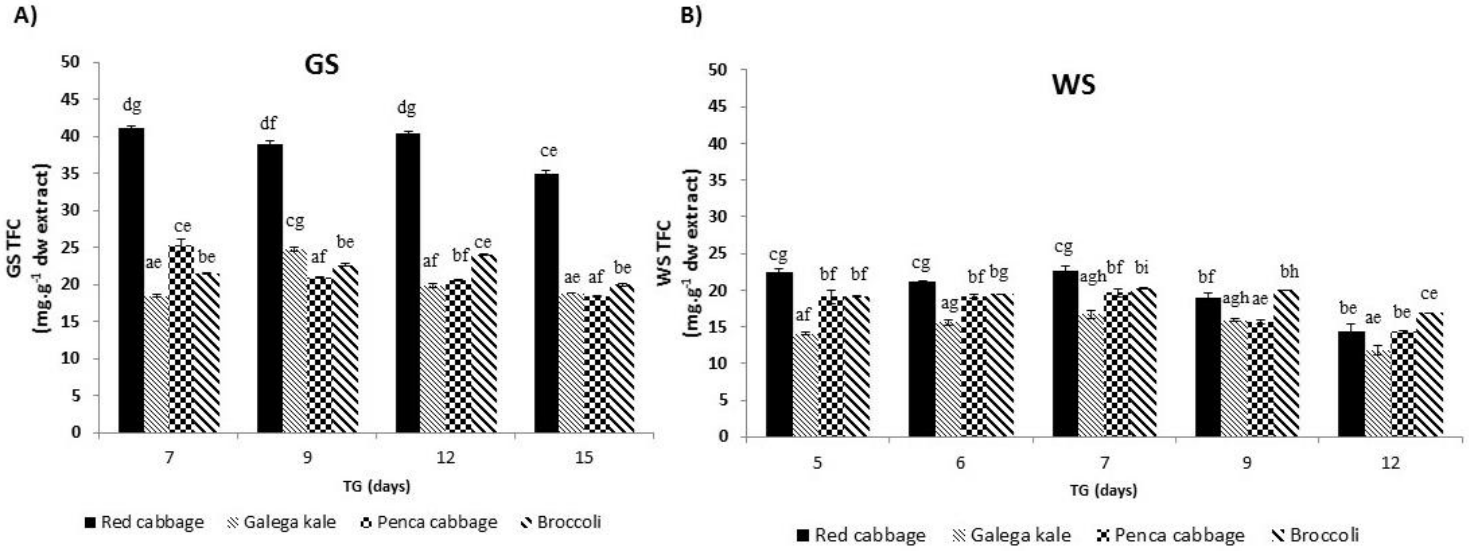


Figure 3.1 Total flavonoid content expressed as quercetin equivalents (mg quercetin.g dried extract-1). Significant differences (p< 0.05) between varieties at the same germination time are represented by different letters between a and d. Significant differences (p< 0.05) in the same variety between different germination times are represented by different letters between e and i.

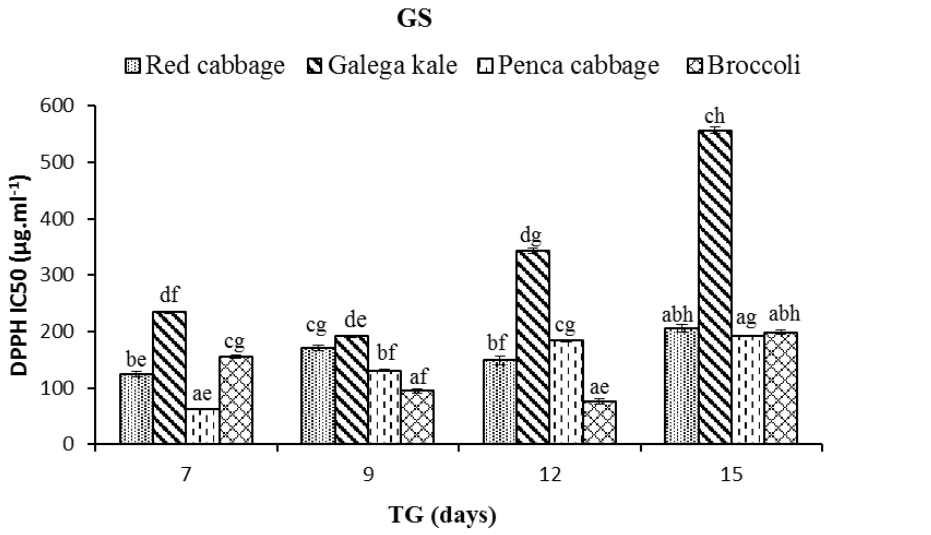
3.3.3. DPPH scavenging activity

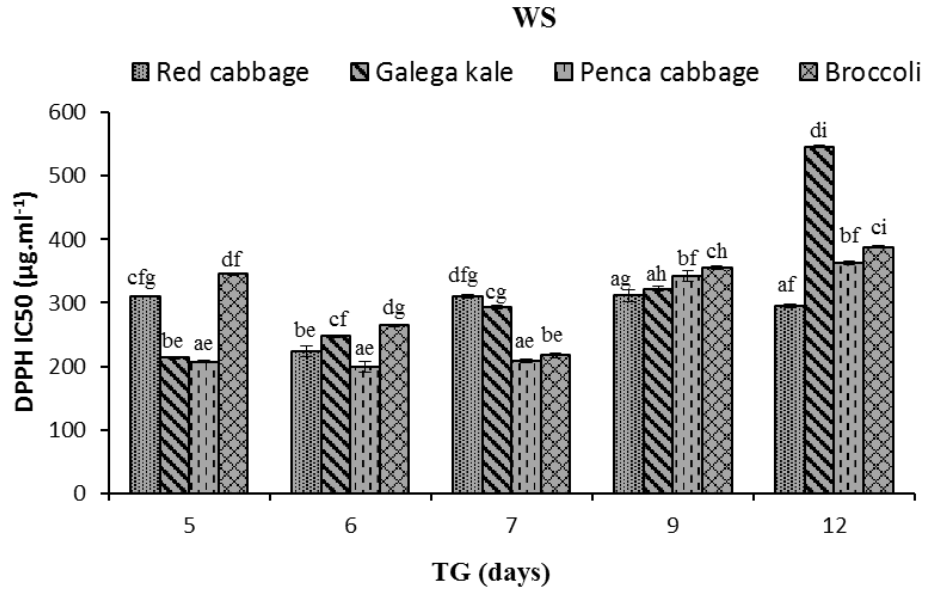
The DPPH radical scavenging activity obtained for the aqueous extracts of sprouts produced in photoperiod (GS) and in darkness (WS) is presented in Figure 3.2A. The results are expressed as IC_{50}. There is a significant light effect ($p<0.05$) on the DPPH scavenging activity of the brassica varieties in study. The sprouting method with a photoperiod (GS) showed always products with higher DPPH scavenging activity than the sprouts developed in darkness (WS). Germination time also influences the scavenging activity of the sprouts. DPPH scavenging activity is statistically different in sprouts with different germination times, with a lower activity at 15th day in GS and at 12th day in WS, exception made for red cabbage (Figure 3.2A). The decrease of the value of this parameter with the germination time followed the pattern verified for the total contents of phenolics and flavonoids (Table 3.1 and Figure 3.1). In general, Galega kale was the variety with less antioxidant potential, regarding the DPPH radical, for the two tested sprouting conditions. It maximum activity was reached at 9th germination day (GS) with the best correlation with TPC (R^2 = 0.999). Penca cabbage and red cabbage showed a maximum DPPH scavenging activity after 7 days of germination, corroborating the results of TPC and TFC. The DPPH scavenging activity was strongly related, in Penca cabbage, to the TPC (R^2 = 0.987) and TFC (R^2 = 0.889). Broccoli GS showed the lowest IC_{50} value at 12th germination day, corresponding to the maximum content of TPC and TFC (correlations of 0.967 and 0.869 respectively).

In WS, once again Penca cabbage and red cabbage presented an analogous behavior with a maximum antioxidant activity at 6 days of germination, with Penca cabbage presenting the highest scavenging capacity (IC_{50} = 198.73 µg.mL^{-1}). In Galega kale the DPPH scavenging capacity decreased significantly ($p<0.05$) throughout the germination time, corresponding to a loss of 61% between the first and the last day. For broccoli WS the best IC_{50} was obtained at 7th day (IC_{50} = 217.2 µg.mL^{-1}) with a decrease of 44% between the 7th and 12th days of germination. A low correlation in the WS between DPPH scavenging

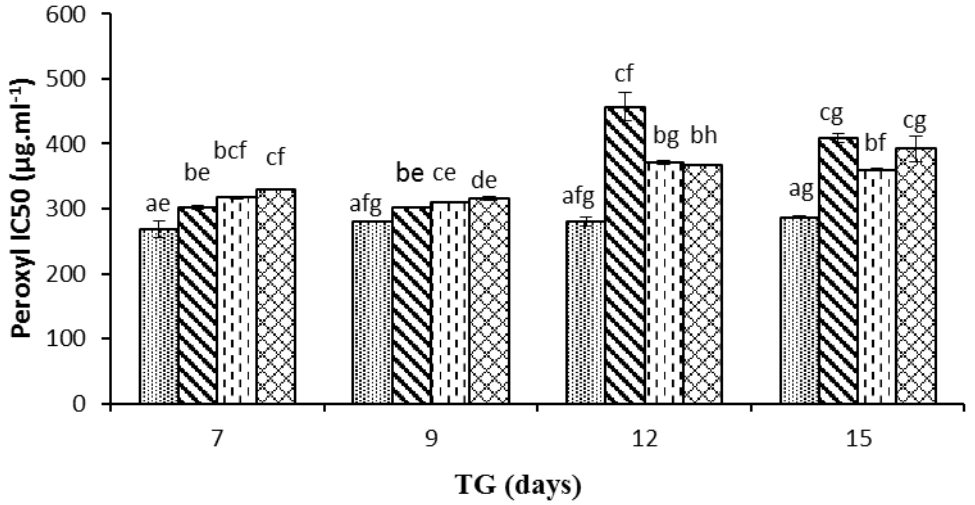
activity and TPC and TFC was also verified, being broccoli (a correlation of 0.924 with TPC) and Penca cabbage (correlation of 0.927 with TPC and 0.975 with TFC) the varieties with better correlations. Similar results were also observed by Dueñas, Hernández, Estrella, and Fernández (2009) in darkness grown sprouts of other species. An antioxidant activity increase, as trolox equivalent antioxidant capacity values, in the germination period was also observed for other species (Frias, Miranda, Doblado, & Vidal-Valverde, 2005; Lin & Lai, 2006) as a result of the biochemical metabolism of seeds during germination.

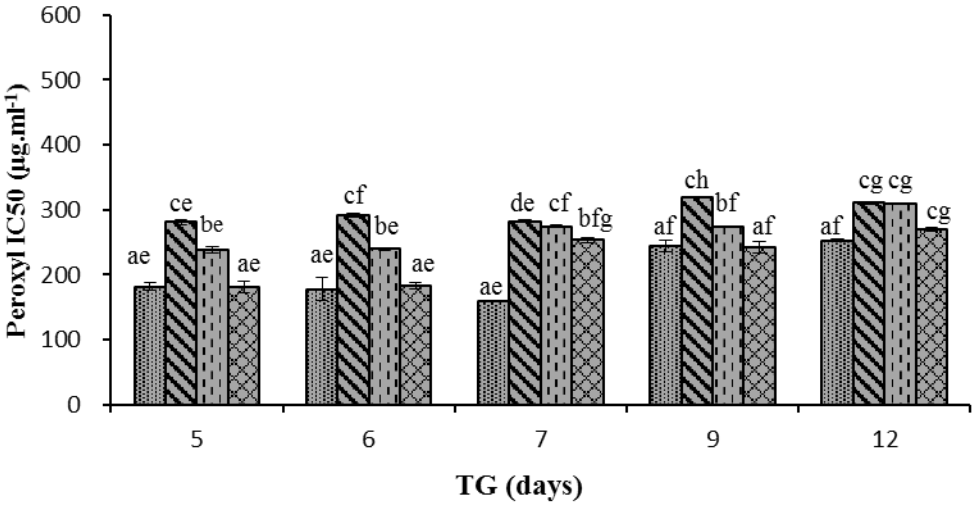
A


GS
Red cabbage
Galega kale
Penca cabbage
Broccoli
DPPH IC50 (µg.ml-1)
600
500
400
300
200
100
0
be
df
ae
cg
cg
de
bf
af
bf
dg
cg
ae
abh
ch
ag
abh
7
9
12
15
TG (days)


WS
Red cabbage
Galega kale
Penca cabbage
Broccoli
DPPH IC50 (µg.ml-1)
600
500
400
300
200
100
0
cfg
be
ae
df
be
cf
ae
dg
dfg
cg
ae
be
ag
ah
bf
ch
af
di
bf
ci
5
6
7
9
12
TG (days)

B


Peroxyl IC50 (µg.ml-1)
600
500
400
300
200
100
0
ae
be
bcf
cf
afg
be
ce
de
afg
cf
bg
bh
ag
cg
bf
cg
7
9
12
15
TG (days)


Peroxyl IC50 (µg.ml-1)
600
500
400
300
200
100
0
ae
ce
be
ae
ae
cf
be
ae
ae
de
cf
bfg
af
ch
bf
af
af
cg cg
cg
5
6
7
9
12
TG (days)

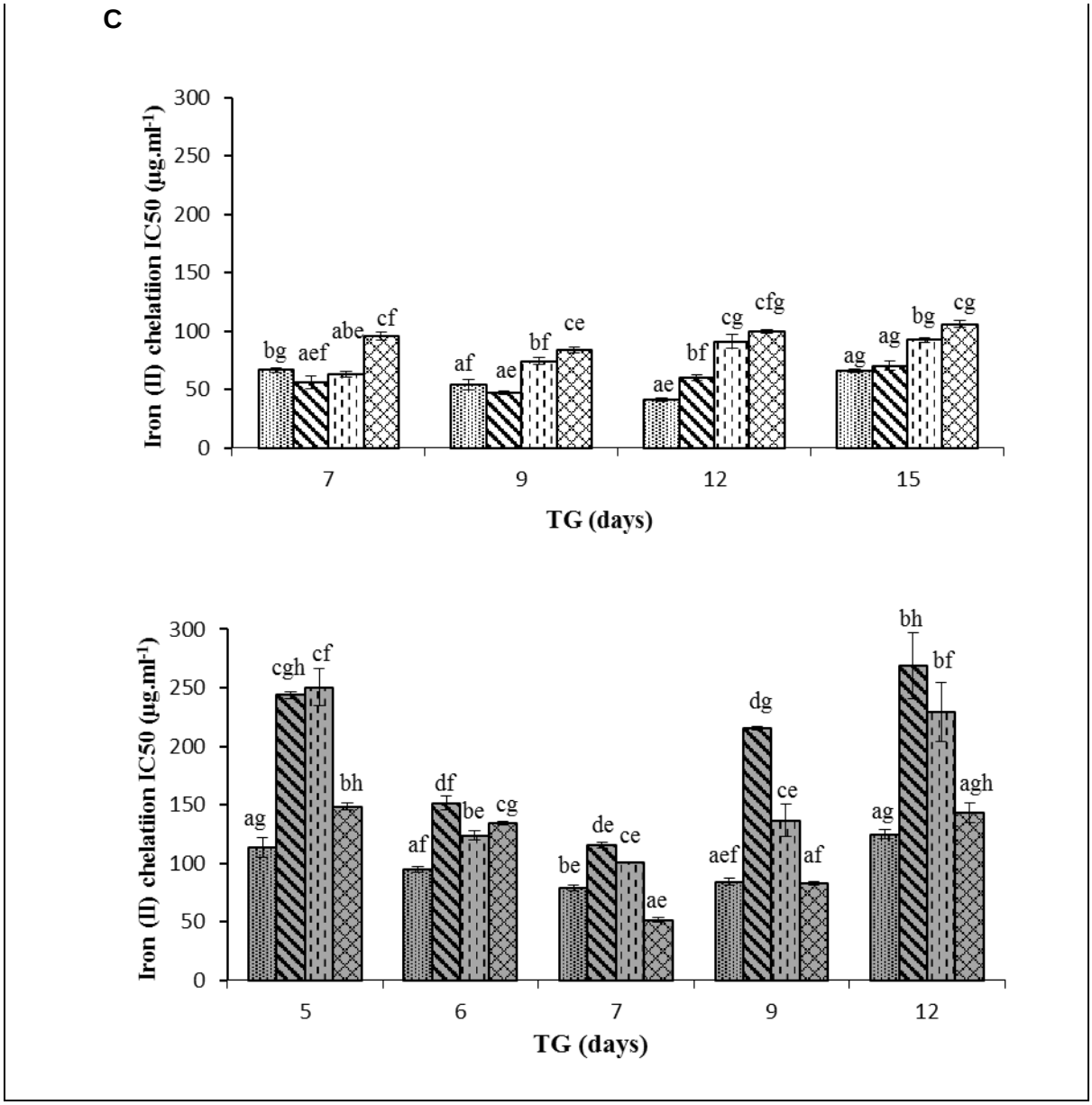


Figure 3.2 Antioxidant capacity of Brassica sprouts expressed as IC50 (lg/mL): A – DPPH free radical-scavenging activity; B – Peroxyl radical Scavenging activity; C – Ferrous ion chelating power. Significant differences ($p < 0.05$) between varieties at the same germination time are represented by different letters between a and d. Significant differences ($p < 0.05$) in the same variety between different germination times are represented by different letters between e and i.

3.3.4. Hydroxyl radical scavenging

Among ROS the hydroxyl radical is the most unstable and reactive and shows a great oxidative power, combining rapidly with almost all molecules in its immediate vicinity (Sousa et al., 2009). As the most reactive ROS, hydroxyl radical can cause several

biological consequences, including mutation, cell death, carcinogenesis and aging (Ragu et al., 2007). Therefore the consumption of food with the ability of scavenging this radical could help to control its harmful effects. The sprouts of all studied Brassicaceae varieties revealed to be $HO^{\cdot}$ radical scavengers in a concentration dependent manner, being GS significantly more active than WS ($p<0.05$). As shown in Table 3.2, the hydroxyl radical scavenging ability in GS decreases in the sequence red cabbage > Penca cabbage > broccoli > Galega kale and in WS behaved as follows: Galega kale > red cabbage > Penca cabbage > broccoli. In general Galega kale GS presents a similar behavior as the reported to the DPPH radical scavenging capacity but in WS was the variety with higher capacity to scavenge the hydroxyl radical. Although significant differences were observed among the studied varieties ($p<0.05$), red and Penca cabbage GS presents closer global average values of IC_{50} (221.46 and 232.10 µg.mL-1, respectively). Broccoli clearly presented a lower ability to scavenging hydroxyl than DPPH radical in the two sprouting methods.

Some compounds can redox cycling the metal ion required for hydroxyl generation, increasing its production and, consequently deoxyribose degradation (Li & Xie, 2000). If ascorbate is not present in the reaction mixture but pro-oxidant compounds, hydroxyl generation is increased. In order to evaluate the pro-oxidant potential of the sprouts extracts, a methodology omitting ascorbic acid from the reaction mixture was implemented and the ability to reduce the Fe^{3+}– EDTA complex tested. It was found that all the extracts tested do not present pro-oxidant activity in the tested concentrations. Sousa et al. (2008) found some pro-oxidant capacity in *B. oleracea* var. *costata* for concentrations below 250µg/mL but for the lowest concentration tested (200µg/mL) brassica sprouts did not present any pro-oxidant capacity, being one more advantage of the consumption of this type of products.

Examining the effect of the germination time on the capacity of sprouts extracts to deactivate the hydroxyl radical it was verified that GS are significantly more active ($p<0.05$)

than WS. The behavior of GS in relation to hydroxyl radical is similar to the observed for the DPPH radical. Red cabbage and Penca cabbage GS presented at the 7th day of germination a maximum antioxidant capacity (IC_{50} = 191 and 204 µg.mL^{-1}, respectively), whereas in Galega kale the maximum occurs at the 9th day (IC_{50} = 219 mg.mL^{-1}) and in broccoli at the 12th day of germination (IC_{50} = 176 µg.mL^{-1}). Broccoli had the lowest IC_{50} value achieved in the GS and it was verified a 38% loss of antioxidant capacity from the day 12th to the 15th. In the case of the other varieties in evaluation, Penca cabbage and red cabbage had a loss of 23% and 31%, respectively, similar to the presented by Galega kale (30%).

WS, though with lower capacity to scavenge hydroxyl radicals, express the maximum scavenging values in the 6th and 7th days of germination, except in broccoli which maximum antioxidant activity is expressed at 5th day, decreasing significantly from this period (43%). The decrease of antioxidant activity over the germination time is in accordance with the results reported by several investigators. In fact at initial germination stages several compounds may serve as radical scanvengers or antioxidants, while later they can become part of the structural framework of the growing plant, as precursors of lignin or lignan structures (De Ascensao & Dubery, 2000) and lose some of their antioxidant efficiency.

3.3.5. Peroxyl radical-scavenger activity

Peroxyl radical is the predominant free radical found in lipid oxidation in foods and biological systems (Prior, Wu, & Schaich, 2005). Accordingly, the consumption of food with peroxyl scavenging activity could help to control its damaging effects. Figure 3.2B shows the results obtained in the ROO$^{\bullet}$ scavenging assay. The results obtained in this study demonstrate that ROO$^{\bullet}$ is effectively scavenged by all tested extracts. It is pointed out that, contrarily to the radicals previously discussed, WS presented a higher antioxidant capacity against peroxyl radical than GS (Figure 3.2B).

Light decreases significantly this capacity, leading to a higher reactivity of the germinated seeds produced in darkness. Many antioxidants that react quickly with peroxyl radicals may react slowly or may even be inert to other radicals like the long-lived nitrogen radical DPPH due to steric inaccessibility (Huang et al., 2005; Prior et al., 2005) and the antioxidants presented in the WS seem particularly reactive to peroxyl radicals. GS sprouts harvested at 7^{th} and 9^{th} germination days presented higher antioxidant activity than the older ones. Red cabbage, despite the apparent highest peroxyl scavenging capacity at the 7^{th} day, presented no statistically differences along the period in evaluation.

In WS, once again red cabbage has the highest antioxidant capacity, followed by broccoli, Penca cabbage and Galega kale, the latter always as the Brassica variety with less capacity to scavenge peroxyl radical. The best germination time to benefit of greatest antioxidant capacity in broccoli, Galega kale and Penca cabbage sprouts produced in darkness was precisely the first harvest time (5 days). Red cabbage WS had a distinct behavior being more efficient at the 7^{th} day. A longer germination time seems to decrease the antioxidant capacity of the WS sprouts, expressed as IC_{50} at levels of 37%, 33% and 23% for red cabbage, broccoli and Penca cabbage, respectively. Galega kale had the lowest antioxidant capacity and suffered the lowest loss (about 12%). This loss of antioxidant activity along the germination time was not identical in the GS, highlighting Galega kale and red cabbage as the varieties with respectively highest (34%) and lowest (7%) losses. As far as we know there are no reports on the scavenging activity levels of *Brassica* mature plants or sprouts against peroxyl radicals using the rate of lysozyme activity assay. Anthocyanin-rich samples like red cabbage sprouts exhibited the most potent antioxidant activity against this radical.

Table 3.2 Hydroxyl radical scavenging assay. Hydroxyl radical scavenging activities of *Brassica* sprout extracts and the reference compound mannitol. The data represent the percentage inhibition of deoxyribose degradation expressed as IC50.

TG (days)	Light effect	IC_{50} Hydroxyl radical (µg/ml)			
		Red cabbage	Galega kale	Penca cabbage	Broccoli
7	GS	191.14±0.65^{a,e}	232.69±3.68^{b,e}	203.96±11.32^{a,e}	282.89±1.81^{c,f}
9		201.93±5.42^{a,e,f}	219.41±3.96^{a,e}	206.98±4.2^{a,e}	282.88±0.75^{b,f}
12		213.35±2.62^{b,e,f}	298.74±3.65^{d,f}	264.54±0.58^{c,f}	175.79±0.82^{a,e}
15		279.41±1.31^{b,g}	314.83±1.49^{c,g}	252.9±0.035^{a,f}	283.74±1.07^{b,f}
5	WS	647.36±2.62^{b,f}	616.5±5.8^{a,g}	619.23±2.72^{a,e}	615.87±3.65^{a,e}
6		576.21±1.27^{a,e}	594.24±3.51^{a,f}	583.62±2.56^{a,e}	759.66±6.52^{b,f}
7		643.58±2.87^{c,f}	542.66±1.21^{a,e}	592.39±2.03^{b,e}	886.71±7.3^{d,g}
9		813.79±8.07^{a,b,g}	770.06±1.58^{a,h}	938.78±7.15^{c,f}	864.8±6.76^{b,c,g}
12		797.57±6.63^{a,g}	847.62±7.28^{b,i}	942.01±4.87^{c,f}	1069.51±7.22^{d,h}

Mannitol as a positive control presented a scavenging capacity of 50.06%. The results are mean ± S.D. of three parallel measurements. Differences between varieties at the same germination time are represented by letters between a and d, and the same letter means non-significant differences ($p< 0.05$). Differences in the same variety between different germination time are represented by letters between e and i, and the same letter means non-significant differences ($p< 0.05$).

3.3.6. Ferrous ion-chelating ability

Among the transition metals, iron is known to be the most important lipid pro-oxidant due to its high reactivity (Gulcin, Bursal, Sehitoglu, Bilsel, & Goren, 2010a; Gülçın, Kıreççı, Akkemık, Topal, & Hısar, 2010). Free iron plays an important role in production of oxygen derived free radicals by Fenton reaction (Halliwell, 1997). These free radicals may be involved in the progression of several dysfunctions namely human cardiovascular disease (Gülçin et al., 2010).

Fe^{2+} (ferrous ion) is the most powerful pro-oxidant among the various species of metal ions. Ferric ions (Fe^{3+}) also produce radicals from peroxides although the rate is tenfold less than of ferrous ion (Fe^{2+}) (Koksal, Gulcin, Beyza, Sarikaya, & Bursal, 2009).

One way to evaluate the metal-chelating activity of an antioxidant is the absorbance measurement of Fe^{2+}–ferrozine complex after treatment of a ferrous ion solution with sample material. Ferrozine forms a complex only with free Fe^{2+} producing a chromophore with maximum absorbance at 562 nm. The measurement of color reduction allows the estimation of the chelating activity. Therefore, the ability of sprout extracts to chelate Fe^{2+} ions was evaluated, expressed as IC_{50} ($\mu g.mL^{-1}$) and the results are presented in Figure 3.2C. All varieties showed a significant metal chelating capacity, being this effect dependent on photoperiod conditions and germination time. The iron chelating activity of GS was significantly higher ($P < 0.05$) than WS, except for broccoli since its higher activity is achieved in WS for the 7^{th} day of germination. Concerning GS, red cabbage is the variety with the highest chelating capacity (IC_{50} = 57.1$\mu g.mL^{-1}$, 12^{th} day of germination), followed by Galega kale GS (IC_{50} = 58.5 $\mu g.mL^{-1}$, 9^{th} day of germination). Broccoli GS showed about 40% lower activity than red cabbage. As observed for radical scavenging activity, for all studied varieties long germination periods (15 days) corresponded to a lowest Fe^{2+} chelating activity.

Regarding WS, for all *Brassicaceae* varieties the highest chelating activity was observed for the 7^{th} day of germination. For this period Broccoli WS had the highest capacity to chelate Fe^{2+} (IC_{50} = 51.57 $\mu g.mL^{-1}$) followed by red cabbage WS (IC_{50} = 78.54 $\mu g.mL^{-1}$). Penca cabbage and Galega kale WS had the lowest activity, showing similar IC_{50} values (115.5 and 100.6 $\mu g.mL^{-1}$, respectively). Interestingly, after an increase of chelating activity between the 5^{th} and the 7^{th} of germination, a decrease of this activity is observed for older sprouts. This loss of activity with longer germination times is smaller in red cabbage (37%) than with Galega kale (56%), Penca cabbage (57%), and broccoli (64%).

3.3.7. Correlation analysis of the measurements

In order to evaluate the relationship between the content of total phenolics, total flavonoids, and the antioxidant activity expressed by the different assays performed, a Pearson's correlation coefficient was analyzed and some significant correlations were found.

Although no strong correlations were achieved ($0.8 \leq r < 1$), significant moderate correlations at 0.01 level, between TFC and antioxidant effect measured by the iron chelating activity and hydroxyl and DPPH radicals scavenging activity (0.585, 0.587 and 0.506, respectively) were found. Ebrahimzadeh, Nabavi, and Nabavi (2009) found for some medicinal plants a direct relation between iron chelating activity and the content of active compounds but no correlation was found between phenol and flavonoid contents of an extract and its chelating activity. However, brassica sprout extracts showed a significant correlation between iron chelating activity and TFC. The results corroborate the importance of flavonoid compounds in the antioxidant behavior of the extracts and also show its significant contribution to the total antioxidant capacity of *Brassica* sprouts. Heimler, Vignolini, Dini, Vincieri, and Romani (2006) compared the main phenolics in several *B. oleracea* crops and reported that broccoli and kale varieties exhibit the highest content of both total phenolics and flavonoids, being our sprouts included in these groups, which may justify its antioxidant properties and the correlation with the total content of flavonoids.

Concerning the correlation between the antioxidant activity of the tested extracts and TPC, the peroxyl and DPPH scavenging activities are significantly correlated with the TPC, at the 0.01 level (0.505) and the 0.05 (0.396), respectively. It should be noted that our results are in accordance with the reported by Aires et al. (2011) who found a low Pearson correlation between TFC and DPPH (0.4580) and a moderate positive correlation between TPC and DPPH (0.64). Nevertheless these authors worked with mature Brassica plants instead of sprouts. In our studies a correlation between iron chelating activity and hydroxyl

radical scavenging activity was established. Additionally, correlations between DPPH * hydroxyl radicals (0.606) and between peroxyl* hydroxyl radicals (0.484) were also found.

For a clearer arrangement, the results of the different assays were grouped in a manner that assigned similar behavior using the hierarchical agglomerative cluster analysis. The cluster analysis on the square Euclidean distances between subjects with the aggregation methods of the smallest distance resulted in three clusters, according to the criteria of R^2, explaining 75.4% (Rsq = 0.754) of the total variance (see Figure 3.3).

The DPPH, hydroxyl and Fe^{2+} chelating activity assays are a behavioral cluster neighborhood that differs from the assays performed to assess peroxyl scavenging capacity and a cluster linking TPC and TFC confirms the moderate correlation previously cited.

A discriminant analysis was also performed in order to evaluate the discriminant effect of the analyzed factors (photoperiod, germination time and *Brassica* variety). Germination time demonstrated no discriminant effect on the results, despite the differences between samples previously discussed. Photoperiod had a highly significant ($P < 0.001$) discriminant effect for almost all parameters, except TPC. The hydroxyl scavenging capacity is the most important test in the differentiation of sprouts extracts. The canonical discriminant function reveals that 92% of variance is explained by the photoperiod factor. Given the discriminant effect of photoperiod we also seek to evaluate the influence of this factor in each *Brassica* variety. The application of the discriminant analysis revealed that the varieties are distributed in two main discriminant dimensions and the discriminant effect was highly significant ($P < 0.001$) (Figure 3.4).

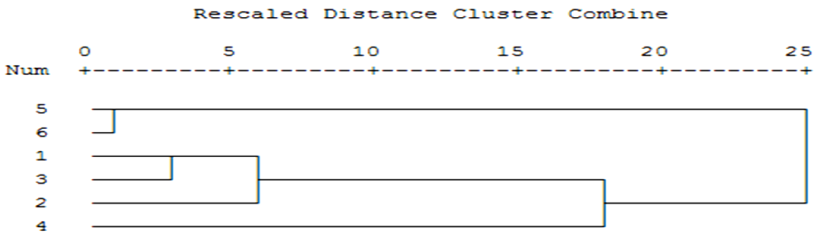


Figure 3.3 Dendrogram of the hierarchical agglomerative cluster analysis based on Pearson correlation similarities indicating the grouping obtained for the results of the antioxidant assays and the flavonoid content of the extracts. Legend: 1 – DPPH radical scavenging; 2 – Ferrous Ion chelating Ability; 3 – Hydroxyl radical scavenging; 4 – Peroxyl radical scavenging; 5 – TPC; 6 – TFC.

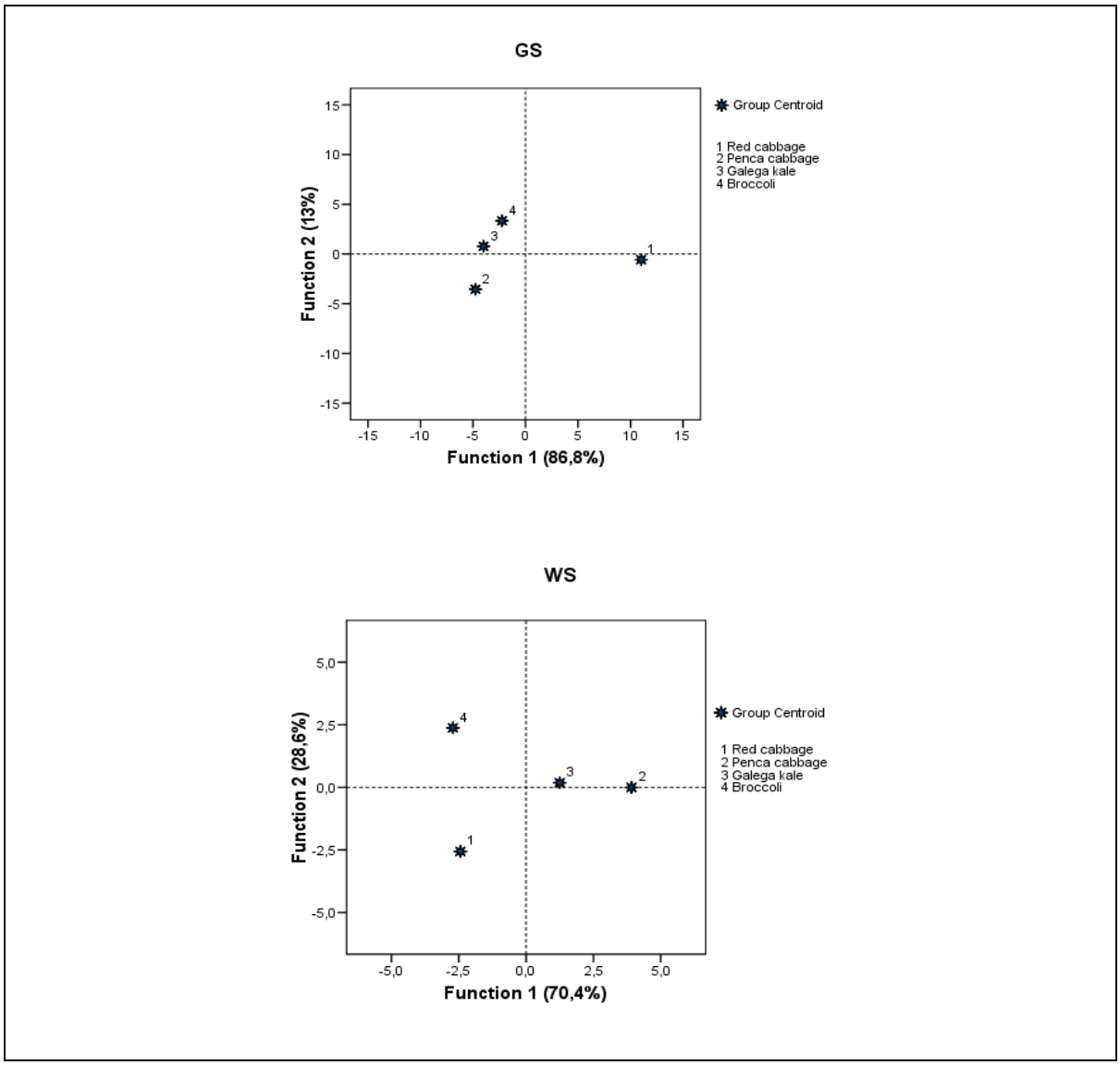


Figure 3.4 Linear discriminant analysis (LDA) results, aggregating all *Brassica* sprout samples.

In GS, TFC showed the better correlation with discriminant function 1 (0.500), which is responsible for 86.8% of the variance observed among varieties, while for the second discriminant function the iron chelating activity is the parameter with best correlation (0.551)

accounting for 13% of the variance obtained. Red cabbage is the variety with main different behavior as was the one with higher antioxidant capacity and red cabbage together with Galega kale and Penca cabbage are the varieties that better defines the function 1. The behavior of WS is different, being the peroxyl scavenging capacity and the iron chelating activity the parameters better correlated with the discriminant function 1 (0.401 and 0.300, respectively) while TPC (0.365) is better correlated with the function 2. These functions accounted for 70.4% and 28.6% of the variance between the varieties for their antioxidant potential. Penca kale and Galega cabbage presented a closer behavior in WS and are the varieties together with broccoli that better defines function 1. Red cabbage again stands out for its distinct characteristics as it corresponds to the sprouted variety with higher antioxidant potential defining function 2.

3.3.8. Conclusions

The antioxidant activity of sprout extracts from *Brassica* varieties was dependent on several factors. The evaluation of the antioxidant activity should be done with different methods in order to avoid being underestimated, since significant differences in phenolic, flavonoid content and antioxidant activities were observed. The correlation discrepancies could be explained, on the basis of differences in the interpretation of the results, by individual methods and/or presence and need of evaluation of other interfering substances (such as ascorbic acid, saccharides and carotenoids). For all studied sprouts, the photoperiod conducted to a less content of flavonoid-type compounds, which were correlated with a significantly lower antioxidant capacity in the WS, except for peroxyl radical scavenging activity. Differences between varieties were also significant, being the selection of a suitable *Brassica* variety of great importance in order to maximize health-promoting properties of sprouts. Green sprouts (GS) from red cabbage were the most interesting's to benefit from high antioxidant capacity. Sprouting resulted in an overall increase in the total phenolic content and antioxidant capacity and although germination time was not a

discriminating factor, higher germination times resulted in lower antioxidant capacity of the sprouts. Because of the small size and biomass, very young sprouts may not be ideal for harvesting, being important to harvest the GS between days 9 and 12 and the WS between 6 and 9 days of germination, since it allows benefiting from its antioxidant potentials. Based on these analyses, it can be suggested that *Brassica* sprouts are a good source of antioxidants and germination brought about a sharp rise in natural antioxidant activity, reaching the highest value before the highest germination time tested. Although the red cabbage sprouts gives a higher antioxidant potential, it is also important to value varieties such as Penca cabbage and Galega kale because they are typical varieties from Portugal, historically used in gastronomy and with deeply studied health benefits.

Acknowledgements

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CAPÍTULO 4

Light influence in the nutritional composition of *Brassica oleracea* sprouts

FOOD CHEMISTRY

Submitted

Light influence in the nutritional composition of *Brassica oleracea* sprouts

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Abstract

Brassica sprouts are considered a healthy food product, whose nutritional quality can be influenced by several factors. The aim of this work was to monitor the nutritional composition changes promoted by different sprouting conditions of four varieties of *Brassica oleracea* (red cabbage, broccoli, Galega kale and Penca cabbage). Sprouts were grown under light/darkness cycles and complete darkness. Standard AOAC methods were applied for nutritional value evaluation, while chromatographic methods with UV-Vis and FID detection were used to determine the free amino acids and fatty acids, respectively. Mineral content was analyzed by Atomic absorption spectrometry. Sprouts composition revealed them as an excellent source of protein and dietary fiber. Selenium content was one of the most distinctive feature of sprouts, being the sprouting conditions determinant for the free amino acid and fatty acids profile. The use of complete darkness was beneficial to the overall nutritional quality of the brassica sprouts studied.

Keywords: brassica sprouts, nutritional evaluation, sprouting conditions, minerals, amino acids, fatty acids profile.

4.1. Introduction

Nowadays, the interest in healthy diets has increased. Fruits and vegetables have attracted a great deal of attention due to their functional components and health-promoting effects related with lower cardiovascular disease risk (Djousse, Arnett, Coon, Province, Moore, & Ellison, 2004) and protection against cancer (Hung, et al., 2004). Raw agricultural products provide not only essential nutrients, but also a large number of biologically active compounds, whose consumption plays an important role in the maintenance of health (WHO/FAO, 2003). Nevertheless, the actual numbers of worldwide vegetables consumption is still below the recommendations in many countries. Consumers from developed countries tend to choose the more convenient food ingredients that are easy-to prepare and consume (Lisiewska, Słupski, Skoczeń-Słupska, & Kmiecik, 2009). Is in this scenery that vegetables sprouts have recently gained more attention in the western society, being their consumption very common in Asian countries for many centuries. Sprouting is an inexpensive and simple procedure (Shohag, Wei, & Yang, 2012), comprising the soaking of the seeds until the protrusion of radicle from the seed occurs (Cáceres, et al., 2014; Khalil,et al., 2007). During germination a reactivation of the seed metabolism takes place, promoting the hydrolysis of storage proteins and carbohydrates and the synthesis/accumulation of metabolites with health-promoting properties (Cáceres, et al., 2014). For this, sprouting is associated with the improvement of the nutritive value of seeds (Khattak, et al., 2008; Márton, Mandóki, & Csapó, 2010; Shohag, et al., 2012). Thereby, sprouts can be considered an important, complete, beneficial and functional foods (Shohag, et al., 2012), being many times referred as a good protein source, and an alternative to the expensive and scarce animal protein (Aguilera, et al., 2013; Tarasevicienė, et al., 2009). Sprouts are also recognized as fresh and healthy ingredients, with low caloric value, high biological activity, reduced content of anti-nutritional components (eg. phytic acid and flatulence causing oligosaccharides), higher concentrations and bioavailability of micronutrients (eg. trace minerals) and improved digestibility and sensory properties (Khattak, et al., 2008; Luo, et al., 2013). Due to their nutritional properties, sprouts, especially from germinated grains, are been used in the formulation of baby and geriatric foods (Luo, et al., 2013).

In response to the increased demand of sprouts, a varied supply has been developed at the European and the Asian markets. The most popular are sprouts of adzuki bean, alfalfa, broccoli, buckwheat, clover, mungo bean, mustard, radish, red cabbage and soybean. Among the more consumed sprouts are those from *Brassicaceae* family, one of the major vegetable crops grown worldwide (Ayaz, et al., 2006; Baenas, Moreno, & García-Viguera, 2012). The vegetables from this family are mainly recognized by their glucosinolate content, being also rich in phenolic compounds, vitamins and minerals (Baenas, et al., 2012). The content of each bioactive compound is influence by genetic, environmental factors, and production and storage conditions (Baenas, et al., 2012). Different studies showed drastic changes of the sprouts nutritional profile during germination, showing in this phase a higher content of phenolics (Baenas, et al., 2012; Vale, Cidade, Pinto, & Oliveira, 2014), minerals (el-Adawy, 2002; Luo, et al., 2013; Tarasevicienė, et al., 2009), and also a different profile of proteins (Gulewicz, e al., 2008; Lisiewska, et al., 2009; Tarasevicienė, et al., 2009) and of saturated and unsaturated fatty acids (Márton, et al., 2010).

The growing conditions during sprouting are many times referred as determinant for the nutritional composition of the sprouts (Cáceres, et al., 2014; Khattak, et al., 2008; Tarasevicienė, et al., 2009), being even a distinctive characterist to classify the sprouts into different categories in Japan (artificial or natural light or in dark).

Considering the potential of sprouts as natural functional food (Moreno, Carvajal, Lopez-Berenguer, & Garcia-Viguera, 2006) the aim of this work was to monitor the changes in the nutritional value of sprouts from different *Brassica oleracea* varieties, including varieties traditionally consumed in Portugal, grown in different photoperiod conditions (light and dark cycles *vs* total darkness). The obtained data would contribute to improve the knowledge about the nutritional importance of sprouts intake and to promote their production as native products with a high nutritional value. To our knowledge, no information is available about these sprouts varieties on the Portuguese food composition Table (PFCA).

4.2. Materials and methods

4.2.1. Chemicals and reagents

All chemicals, reagents and solvents use in the proximate analyses and mineral composition determination were from analytical grade purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Acetonitrile and methanol used in HPLC analysis were from HPLC grade and also bought from Sigma Chemical Co. The water used was treated in a Milli-Q water purification system (Millipore, Bedford, MA, USA).

4.2.2. Plant material

Untreated seeds from Broccoli (*B. oleracea* L. var. italica Plenck, cultivar calabrese), Portuguese Galega kale (*B. oleracea* var. acephala DC), Portuguese Tronchuda cabbage (*B. oleracea* L. var. costata DC, landrace Penca da Póvoa) and red cabbage (*B. oleracea* var. capitata f. rubra) were used for sprout production. Broccoli and red cabbage seeds were supplied by Germisem - Sementes Lda. The seeds of the traditionally consumed varieties, Portuguese Tronchuda cabbage (penca cabbage) and Portuguese Galega kale were acquired directly from the producers in Póvoa do Varzim (North of Portugal).

4.2.3. Sprouting

Sprouting was carried out according to the method described by Martinez-Villaluenga, et al. (2010) with slight adjustments. Seeds were previously soaked with sodium hypochlorite solution (0.07%, v/v) for 30 minutes, drained and washed with distilled water until they reached a neutral pH. After, they were soaked in water for 12 hours in darkness, at room temperature with moderated shaking. Followed their placement in individual trays containing vermiculite (10x15x4cm) that were put in a plant growth chamber (Fitoclima 200, Aralab, Rio de Mouro, Portugal) with controlled temperature (25ºC) until germination. Two types of sprouts were produced accordingly to the photoperiod conditions used. For green sprouts (GS) production the seeds were submitted to a photoperiod regime with a cycle of 16 hours of light and 8 hours of darkness; whereas seeds for production of white sprouts (WS) germinated in the darkness. Germination process was carried out in triplicate for each germination condition, with a germination yield over 98%. Sprouts where harvested when reached a commercial size, being frozen at -80ºC, freeze-dried (Scanlaf 110-4 PRO, Lynge, Denmark), ground in a mill (Retsch ZM 200, Haan, Germany) and kept in a desiccator until analysis.

4.2.4. Nutritional Composition analysis

4.2.4.1. Proximate analyses

The dry matter/moisture content, total protein (factor of 6.25), fat, crude fiber and ashes contents were determined accordingly to the (AOAC, 2000) methods. The dry matter content of the seeds was determined before and after germination, by drying them at 105°C to constant weight. Simultaneously the moisture content was also determined. The sprouts

protein content was estimated by the Kjeldahl method, while fat was determined by Soxhlet extraction with petroleum ether. Crude fat content was given with 0.1% accuracy as the mean value of three repetitions. Crude fiber content was assessed by sample digestion in sulfuric acid and sodium hydroxide solutions followed by calcination of the residue. Ash content was determined by sprouts incineration at 600±15 ºC. Nitrogen-free extract (NFE), manly composed of digestible carbohydrates, and other non-nitrogen soluble organic compounds, was calculated according to the principle of difference: amounts of crude proteins, crude fats, crude fiber and crude ash took from 100. All values were presented as a percentage, being the energy value obtained according to (Osborne, 1978), by multiplying the percentage of crude protein, crude fat and NFE by the factor of 4, 9 and 4, respectively.

4.2.4.2. Dietary fiber determination

Total dietary fiber was analyzed by the enzymatic-gravimetric method proposed by Prosky, Asp, Schweizer, DeVries, Furda, and Lee (1994). The method is based on the sequential use of three enzymes (heat-stable α-amylase, protease and amyloglucosidase) under different incubation conditions in order to remove starch and protein components. The residue obtained after precipitation with 95 % ethyl alcohol and filtration (CFS 6 filtration system, Velp Scientific, Usmate, Italy) was divided in two fractions to get residual ashes and residual proteins for corresponding corrections. Total dietary fiber (TDF) was calculated as: [(weight residue – protein – ash – blank)/ weight test portion], being weight residue and weight test portion an average of duplicates.

4.2.4.3. Determination of mineral composition

The content of the main (phosphorous (P), potassium (K), calcium (Ca) and magnesium (Mg)) and trace (iron (Fe), zinc (Zn) and selenium (Se)) minerals were determined in the freeze-dried sprouts samples. Briefly, 1 g was refluxed in a digestion system (Velp DK 42P) for 2 h with 6 mL of 65% HNO_3 under different temperatures (30 min at 50 ºC; 30 min at 80 ºC; 30 min at 150 ºC; and 30 min at 165 ºC) and for 3 h with 4 mL of 70% $HClO_4$ (30 min at 165 ºC; 60 min at 180 ºC; 60 min at 190 ºC; and 30 min at 200 ºC). After cooling, 10 ml of ultrapure water were added to each sample and left to stand for 60 min at 120 ºC. Final volume was adjusted to 50 ml with ultrapure water. Potassium, calcium, magnesium, iron, zinc and selenium were determined in the digested solution by flame-atomic absorption spectrometry (Perkin Elmer AAnalyst 200, Waltham, MA, USA), while phosphorous content was determined according to the 4500-P B. Ascorbic Acid standard Method (Greenberg, Clesceri, & Eaton, 1992) in a UV/VIS spectrophotometer at 670 nm.

4.2.4.4. Free Amino acids

The extraction and purification of free amino acids (AA) was performed according to the method described by Gomes and Rosa (2001). Briefly, 0.2 g of freeze-dried sprouts were extracted twice with boiling methanol (90%) for 2 min under continuous homogenization (Ika Ultra Turrax T_{25}, with S25N-10G dispersing element) at 24 000 rpm. Extracts were centrifuged for 2 min at 4000 rpm and the supernatant transferred into a 10 mL volumetric flask. This step was repeated twice using methanol (70%). Combined supernatants were made up to a final volume of 10 mL with methanol (70%) and kept at –18ºC until analysis. Later, a volume of 2 mL of extract were evaporated and resuspended in 2 ml of 0.1 M HCl. Mini-columns of 1 mL (Chromabond, Macherey-Nagel) were connected to a solid phase extraction vacuum system and first eluted with 0.5 mL of 0.1 M HCl, being then filled up with 1mL (approximately 2 cm) of a cation exchange resin, Dowex (H^+) 50WX8-499 (($C_{10}H_{12}.C_{10}H_{10}.C_8H_8$)x, 69011-20-7, Sigma-Aldrich Chemicals, St Louis, MO, USA). Resuspended extracts were loaded into the columns and washed with 5 mL of 0.1 M HCl. The free amino acids were eluted with 4 x 2.5 mL of 7 M NH_3 (pa grade, Merck, Darmstadt, Germany). The eluted extracts, containing the free amino acids, were evaporated at 35ºC in a Termobloc (T150 P2, Falc Instruments, Italy) and the residue resuspended in 0.3 mL of distilled water, filtered (Spartan 13, 0.2 µm) and kept in vials at -18 ºC until analysis. The free amino acids were determined by HPLC method using a reversed phase C18 column (150mm x 4.6mm (i.d.),Waters, Spherisorb S3 ODS2) and a UV/VIS detector set at 340 nm, after pre-column derivatization with o-phthalaldehyde/2-mercaptoethanol, following the procedure described by Gomes and Rosa (2001). The mobile phase was composed by two eluent mixtures: A – 350 mM Na_2HPO_4. $2H_2O$ and 250 mM propionic acid (1:1)/ acetonitrile/ water (40:8:52); B – acetonitrile/ methanol/ water (30:30:40). The gradient employed was the following: 0 min, 100% A; 9.5 min, 89% A; 11 min, 88% A; 13.6 min, 80% A; 20.4 min, 55% A; 23.4 min, 50% A; 25.4 min, 40% A; 32 min, 0% A; 34 min, 100% A; 37 min, 100% A. A flow rate of 1.3 mL min^{-1} was used in most of the gradient program, being reduced to 0.8 mL min^{-1} between the 25.4 to 34 min. For the identification and quantification of amino acids the external standard methodology after adjustment through regression lines were used.

After quantification of each amino acid present in the extract, an essential amino acids score was calculated according to the FAO/WHO reference amino acid pattern (FAO/WHO, 1985).

$$Amino\ acid\ score = \frac{Test\ amino\ acid\ x100}{Reference\ amino\ acid}$$

4.2.4.5. Fatty acid profile

The fat was extracted from the freeze-dried sprouts with a methanol (0.01% BHT): chloroform solution (1:2) and gravimetric determination accordingly to Borges, Oliveira, Casal, Dias, Conceicao, and Valente (2009). A solution of NaCl 0.9% (0.7 mL) was also added before centrifugation at 3300 *g* for 15 min. The fat extract was first dried under nitrogen flow and then dissolved in 1 mL of hexane before storage at -20ºC. The fatty acid methyl esters (FAME) were prepared by transesterification with boron trifluoride (Sigma Aldrich St. Louis, MO, USA) and analyzed by gas chromatography in a Shimadzu GC-2010 gas chromatograph equipped with a split-splitless injector, a FID detector and an autosampler Shimadzu AOC-20i. The chromatograph was equipped with a CPSil 88 fused silica capillary column (Varian, Middelburg, Netherlands; 50 m x 0.25 mm i.d., 0.19 µm film thickness). Helium was used as gas carrier (120kPa) and separation was achieved with the following temperature program: 5 min at 120°C, increase of 3°C/min from 120°C to 220°C, maintaining 220°C for 10 min. The temperature of the injector and detector was 250°C and 270°C, respectively, the *split* ratio of 1:50 and the injection volume was 1µL. Each sample was analyzed in duplicate. FAME were identified by comparison with a standard mixture (FAME 37, Supelco, Bellefonte, PA, USA) and analyzed using the Shimadzu software GC solution 2.30 (Shimadzu, Columbia). The concentration of each FAME was quantified in relation to the total fatty acids and the results expressed as $g.100g^{-1}$ FA (fatty acids).

4.2.5. Statistical analysis

Data obtained from the study were presented as mean ± standard deviation and the differences between samples and growth conditions were tested by one-way ANOVA followed by post-hoc Tukey comparison tests, using the SPSS 15.0 software (SPSS Inc., Chicago, Illinois, EUA) for Windows. Statistical significance was defined for $p < 0.05$.

4.3. Results and discussion

4.3.1. Nutritional quality of Brassica sprouts

Traditionally, the more popular sprouts are from leguminous plants, however the ready-to-eat sprouts from brassica plants have great potential as high quality foods, showing a great potential as functional foods as was demonstrated in a previous work regarding the characterization of the antioxidant capacity of these sprouts (Vale, et al., 2014). However, the nutritional quality of the sprouts from the selected *Brassica oleracea*

varieties was not fully characterized until now, as well as the influence of growth conditions (eg. light exposure) during sprouting. In this work, different nutrients were assessed to characterize the nutritional quality of the selected sprouts. Their macronutrient composition is presented on Table 4.1. As expected for vegetable products, all sprouts show a high water content (more than 90%), being also noticeable their protein and total dietary fiber content (see Table 4.1). Although the sprouts studied were all from the same species, their varieties was reflected on their composition, showing significant ($p<0.05$) differences between them in all parameters, with exception for the energy value.

Regarding the influence of sprouting conditions tested, i.e., the light exposure, some significant differences ($p<0.05$) on their nutritional content were also found (see Table 4.1). Dry matter losses and consequently increasing of water content are inevitable in germinating seeds, due to imbibing and other physiological processes that take place during germination. One of the basic tasks in seeds germination technology is minimizing dry matter losses, since a higher dry matter content represents a higher nutritional value. Moisture content from Galega kale and Penca cabbage increased in WS having the presence of light a significant effect in the production of sprouts with higher nutritional value. Similar results were found by Khattak, et al. (2008) where darkness resulted in maximum mean values for moisture content of chickpea sprouts. In red cabbage and broccoli there were no differences between moisture content of GS and WS. Ash content, followed the inverse trend of moisture in kale and Penca cabbage sprouts, with GS having a higher ash percentage; however, in Penca cabage the differences were non-significant. These results are in agreement with those presented by Khattak, et al. (2008) that found a higher ash content in irradiated sprouts. In Red cabbage the behavior was distinct from the other varieties, showing a significant 17% increase in the ash content when sprouts germinated in the darkness. Regarding the variation of protein between GS and WS, this was also higher in WS from Red cabbage (more 10% than in GS) and broccoli (more 7% than in GS). In the other hand, the protein content of Penca cabagge sprouts has suffered a slight decreased (4%) when germinated under darkness. Galega kale sprouts were the variety with the highest mean protein content (see Table 4.1). In relation to crude fiber, Penca cabbage was the variety with higher content, increasing 21% from GS to WS. Although only Penca cabbage and Broccoli sprouts showed a significant ($p<0.05$) variation of their fat content between GS and WS, these were the most intense, increasing 30% in Penca cabbage and 28% in Broccoli sprouts grown under darkness. It should be noted that fat content did not differ significantly between the varieties grown in darkness, presenting a mean of total fat content of 9.4%. The absence of light determined also a higher content of

total dietary fiber in all varieties, showing Galega kale and Penca cabbage the highest mean values (38.2% and 38.3% respectively). The increase between GS and WS was of 19% for Galega kale and 21% for Penca cabbage sprouts. A general increase in the level of TDF of seeds from different species germinated in darkness was also observed by Zieliński, Frias, Piskuła, Kozłowska, and Vidal-Valverde (2005). The TDF content found in WS from Galega kale and Penca cabbage were similar to the TDF values found for mature cabbage plants (around 39.6 %) (Khanum, Siddalinga Swamy, Sudarshana Krishna, Santhanam, & Viswanathan, 2000). In this case, the consumption of sprouts could represent a better option as sprouts are usually consumed raw, avoiding the TDF losses associated to cooking processes necessary to prepare mature cabbages, that could represent a TDF loss of 5-10% (Khanum, et al., 2000). The NFE content, representing the digestible carbohydrates fraction, differed significantly between GS and WS, presenting the GS higher mean values, with exception of Galega kale. Broccoli GS was the variety with the highest NFE value (46.07%) showing losses of 17% in the WS. The individual variation of the different components evaluated was not reflected in the energy value of the sprouts, with exception for the Red WS cabbage sprouts that revealed more 4% of energy than GS (see Table 4.1).

Table 4.1 Content of water of fresh sprouts and ash, protein, fat, fiber, dietary fiber (TDF), nitrogen free extract (NFE) and energetic value (Kcal.100g^{-1}) of freeze dried *Brassica* sprouts produced under light cycles (GS) and under dark conditions (WS).

Variety	Water (%)	Crude ash (%)	Total protein (%)	Total fat (%)	Crude fiber (%)	TDF (%)	NFE (%)	Kcal.100g^{-1}(dw)
Red cabbage								
GS	93.67±0.21 ^{b}	16.25±0.06 ^{b*}	26.95±0.46 ^{b*}	8.53±0.15 ^{b}	9.77±0.04 ^{a*}	25.04±0.06 ^{a*}	38.50±0.58 ^{ab*}	338.53±0.85 ^{a*}
WS	93.18±0.26 ^{a}	19.62±0.46 ^{c*}	29.95±0.39 ^{bc*}	9.10±0.49 ^{a}	*10.63±0.32 ^{a}	29.02±0.13 ^{a*}	31.79±0.60 ^{a*}	324.51±5.39 ^{a*}
Galega kale								
GS	92.70±0.24 ^{a*}	15.30±0.62 ^{ab*}	30.27±0.52 ^{c}	7.84±0.45 ^{ab}	10.11±0.31 ^{a}	30.76±0.08 ^{c*}	36.48±0.31 ^{a}	337.57±5.74 ^{a}
WS	94.53±0.30 ^{b*}	13.18±0.36 ^{a*}	31.29±0.20 ^{c}	8.74±0.60 ^{a}	10.08±0.24 ^{a}	38.15±0.57 ^{c*}	36.48±0.33 ^{b}	350.66±5.20 ^{b}
Penca cabbage								
GS	93.75±0.29 ^{b*}	15.14±0.44 ^{ab}	27.49±0.21 ^{b*}	6.80±0.19 ^{a*}	10.18±0.40 ^{a*}	30.15±0.16 ^{c*}	40.39±0.77 ^{b*}	332.72±4.26 ^{a}
WS	94.55±0.15 ^{b*}	14.45±0.07 ^{ab}	26.45±0.38 ^{ab*}	9.75±0.48 ^{a*}	12.83±0.23 ^{b*}	38.27±0.08 ^{c*}	39.72±0.08 ^{b*}	339.65±3.62 ^{ab}
Broccoli								
GS	93.56±0.17 ^{ab}	13.79±0.20 ^{a*}	22.97±0.21 ^{a*}	7.28±0.73 ^{ab*}	9.89±0.24 ^{a}	27.84±0.47 ^{b*}	46.07±0.37 ^{c*}	341.68±5.38 ^{a}
WS	93.13±0.28 ^{a}	15.74±0.82 ^{b*}	24.63±0.33 ^{a*}	10.01±0.38 ^{a*}	9.81±0.19 ^{a}	32.13±0.58 ^{b*}	39.12±0.62 ^{b*}	347.85±5.91 ^{b}

Values are means (n = 9) ± SD expressed on dry weight basis for crude ash, protein, fat, fiber, TDF and energy, and on fresh weight basis for water content.
Means not sharing a common letter in a column are significantly different at $p<0.05$.
***** Means significant differences ($p<0.05$) between GS and WS.

4.3.2. Mineral composition

Sprouts composition in macro-(phosphorous (P), potassium (K), calcium (Ca) and magnesium (Mg)) and micro-minerals (iron (Fe), zinc (Zn), selenium (Se)) is presented in Table 4.2. The sprouts analyzed showed to be a good source of potassium and calcium, especially Galega kale GS that had the highest content (approximately 15 $mg.g^{-1}$(d.w) and 7 $mg.g^{-1}$(d.w), respectively). Red cabbage presented the highest content of phosphorous and magnesium (about 10 $mg.g^{-1}$(d.w) (GS) and 7 $mg.g^{-1}$(d.w) (WS), respectively). The mineral content found in these sprouts was, in general, higher than the values described for kale, broccoli and cabbage mature plants, which makes sprouts a better dietary source of these minerals (Anunciação, Leao, de Jesus, & Ferreira, 2011; Jahangir, Kim, Choi, & Verpoorte, 2009). It should be noted that in the case of kale sprouts, both GS and WS had higher levels of Mg and P, while K content was only higher than the content found in mature plants in GS sprouts (Jahangir, et al., 2009). Light exposure during sprouting had a significant ($p<0.05$) influence for almost all minerals distribution in sprouts, exception made for selenium in broccoli sprouts. Broccoli and Penca cabbage GS showed the lower macronutrient concentration, however, for the other varieties (Red cabbage and Galega kale), light exposure resulted in a higher macro-mineral content, especially potassium and calcium. Exceptions were observed for phosphorous in Galega kale and magnesium in Red cabbage, since WS were richer in these macrominerals. Regarding the micro-mineral content present in the sprouts, the global mean concentration of Fe and Se was also higher in GS (2 $mg.g^{-1}$ (d.w) and 0.2 $\mu g.g^{-1}$(d.w), respectively). In general, light exposure during sprouting had a positive ($p<0.05$) effect on micro-minerals concentration. The most abundant micro-mineral analyzed was Fe followed by Zn. Comparing Se content found in the sprouts, these were in the same range of the values reported by other works for brassica mature plants (Manchali, Chidambara Murthy, & Patil, 2012). Selenium is a well-known anticancer agent (Abdulah, Miyazaki, Nakazawa, & Koyama, 2005) and there are epidemiological studies showing that cruciferous vegetables (Kolonel, Hankin, Whittemore, Wu, Gallagher, Wilkens, et al., 2000) and selenium may reduce the incidence of prostate cancer, making the Se content of sprouts one important trait to promote their consumption as healthy foods.

Table 4.2 Mineral composition of *Brassica* sprouts produced under light cycles (GS) and under dark conditions (WS).

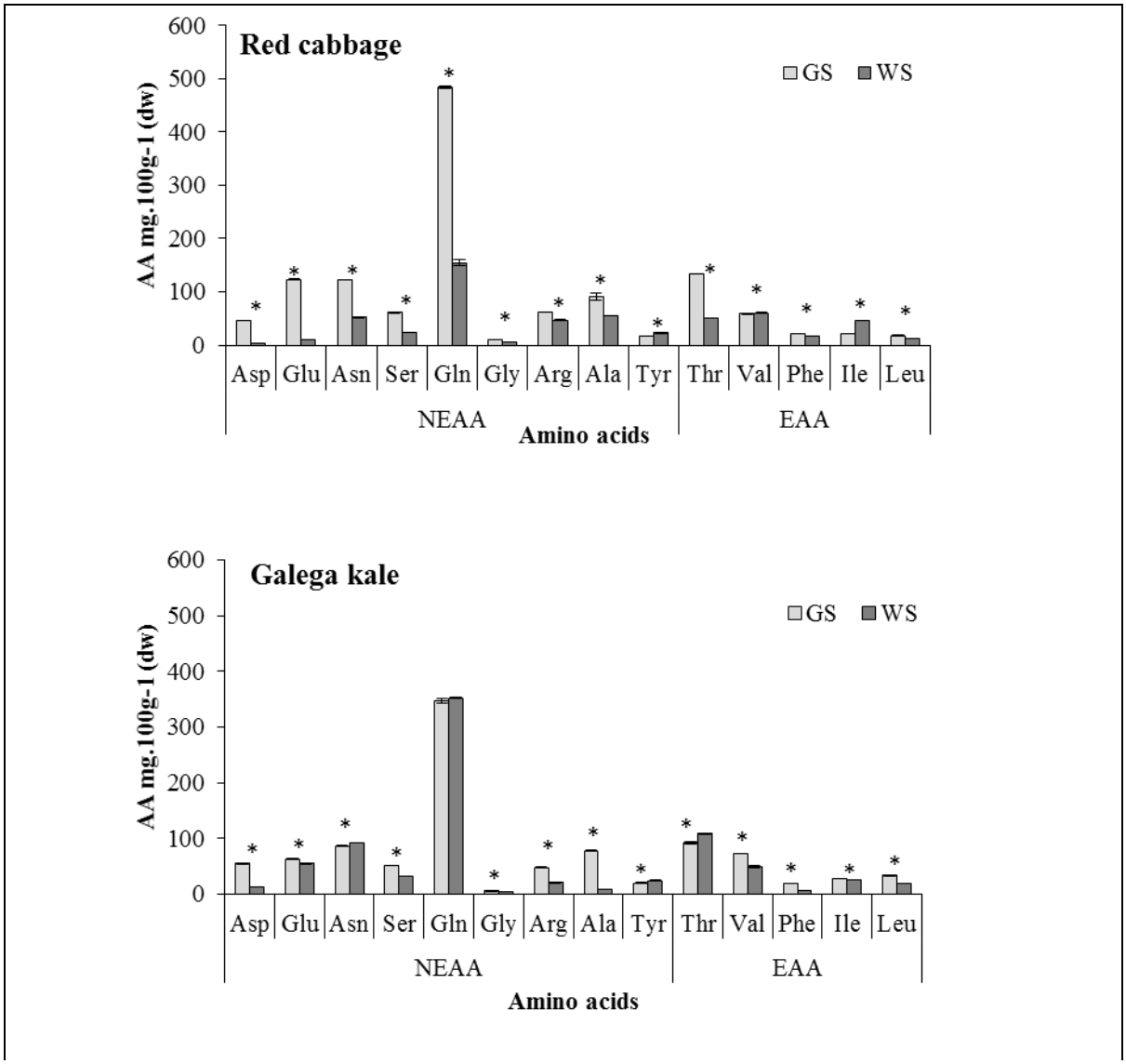
	Red cabbage	Galega kale	Penca cabbage	Broccoli
			mg.g^{-1} (d.w)	
Phosporous				
GS	9.70±0.56 ^{c*}	8.38±0.30 ^{b*}	8.94±0.37 ^{bc*}	7.40±0.35 ^{a*}
WS	8.80±0.15 ^{a*}	10.37±0.40 ^{b*}	10.58±0.12 ^{b*}	8.17±0.28 ^{a*}
Potassium				
GS	13.21±0.37 ^{ab*}	14.62±0.35 ^{b*}	14.00±0.28 ^{ab*}	13.04±0.47 ^{a*}
WS	12.87±0.71 ^{a*}	11.73±0.80 ^{a*}	14.79±0.55 ^{a*}	19.47±0.18 ^{b*}
Calcium				
GS	7.05±0.18 ^{c*}	7.13±0.17 ^{c*}	5.98±0.22 ^{b*}	5.53±0.13 ^{a*}
WS	6.78±0.19 ^{b*}	6.23±0.14 ^{a*}	6.44±0.10 ^{ab*}	6.55±0.17 ^{ab*}
Magnesium				
GS	5.96±0.0.26 ^{b*}	4.63±0.13 ^{a*}	5.01±0.14 ^{b*}	5.44±0.12 ^{a*}
WS	6.57±0.21 ^{c*}	4.73±0.18 ^{a*}	4.65±0.13 ^{a}	5.47±0.20 ^{b*}
Iron				
GS	2.77±0.31 ^{b*}	1.70±0.40 ^{a*}	2.96±0.41 ^{b*}	1.90±0.20 ^{a*}
WS	3.47±0.83 ^{c*}	1.45±0.11 ^{ab*}	1.26±0.40 ^{a*}	2.11±0.60 ^{b*}
Zinc				
GS	0.05±0.00 ^{ab*}	0.06±0.02 ^{ab*}	0.070±0.02 ^{b*}	0.048±0.00 ^{a*}
WS	0.06±0.00 ^{b*}	0.07±0.01 ^{c}	0.054±0.01 ^{a}	0.068±0.02 ^{c*}
			µg.g^{-1} (d.w)	
Selenium				
GS	0.23±0.06 ^{b*}	0.22±0.00 ^{b*}	0.15±0.03 ^{a*}	0.16±0.02 ^{a}
WS	0.15±0.03 ^{b*}	0.14±0.02 ^{ab*}	0.10±0.03 ^{a*}	0.23±0.05 ^{c}

Values are means (n = 9) expressed on dry weight basis
Letters means significant differences (p< 0.05) between varieties for each mineral element;
***** Means significant differences (p<0.05) between GS and WS.

4.3.3. Free amino acid content of sprouts

Figure 4.1 summarizes the total free amino acids (AA) content found in sprouts from the four varieties studied. The sprouts amino acid profile comprised 14 constituents, 5 of which were essentials AA (threonine, valine, phenylalanine, isoleucine, leucine) and one, arginine, which is semiessential, whose content in sprouts was lower than the concentration found for mature Penca cabbage plants (Oliveira et al., 2008). Photoperiod influenced significantly (p<0.05) the AA profile of all sprouts (see Figure 4.1). Galega kale GS showed

higher content in ten AA, Red cabbage GS in eleven AA and penca cabbage GS in thirteen AA, including all essential AA, whereas Broccoli WS were richest in AA, with exception for phenylalanine and leucine content. The most abundant AA in the studied sprouts was glutamine, a nonessential AA, whose predominance in the AA profile was also found in kale (Ayaz et al., 2006) and in primary and secondary inflorescences of 11 broccoli cultivars (Gomes & Rosa, 2001). On the other hand, (Oliveira et al., 2008) reported arginine as the most abundant AA in mature Penca cabbage plants. Glutamine ranged from 126.4 ± 1.4 mg.100g^{-1} (d.w.) in Broccoli GS to 483.5 ± 1.5 mg.100g^{-1}(d.w.) in Red cabbage GS, representing 35% and 38% of the total free amino acid content, respectively. In relation to WS, glutamine content ranged from 155.0 ± 5.5 mg.100g^{-1}(d.w.) in Red cabbage to 526.1 ± 6.5 mg.100g^{-1}(d.w.) in Broccoli, contributing to 28% and 46% of the total AA. Glutamine content was even more expressive in Penca cabbage WS, representing 49% of the total AA profile.



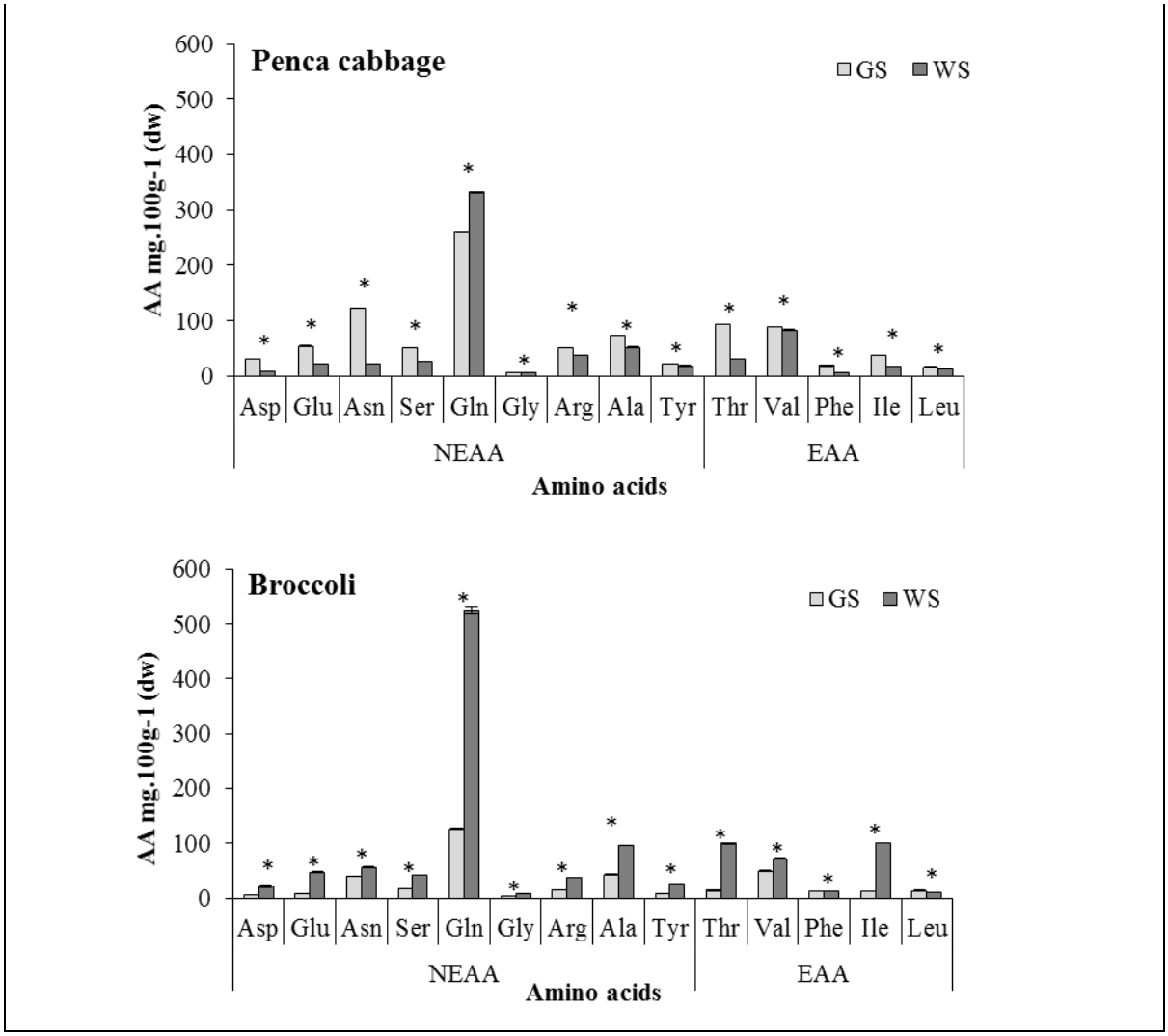


Figure 4.1. Amino acid composition of four Brassica sprouts varieties produced under the light (GS) and under darkness (WS). Mean value ± standard deviation relative to dry weight (d.w.), n=3; * means significant differences ($p<0.05$) between GS and WS. Abbreviations of amino acids: EAA- essential amino acids, NEAA- non essential amino acids, asp, aspartic acid; glu, glutamine; Asn, asparagine; ser, serine; gln, glutamine; gly, glycine; thr, threonine; arg, arginine; ala, alanine; tyr, tyrosine; val, valine; Phe, phenylalanine; ile, isoleucine; leu, leucine.

Regarding only the essential AA, threonine was the most abundant in GS of Red cabbage, Galega kale and Penca cabbage, representing 11%, 9% and 10% of the total AA, respectively, and also in WS of Galega kale and Broccoli (13% and 9%, respectively). However, isoleucine occurred at similar concentration (9%) in WS of Broccoli, being valine the most abundant essential AA in Red cabbage and Penca cabbage WS. The essential

AA were present in higher proportion in WS of Red cabbage and Galega kale representing 36% and 27% of the total AA, against 21% and 26% in GS, whereas in Penca cabbage and Broccoli the higher proportion occurred in GS contributing 29% to the total AA, against 24% and 26% in WS.

The nutritional quality of sprouts AA profile was evaluated by comparing the percentages of the essential amino acids with the values reported by World Health Organization (WHO) (FAO/WHO/ONU, 1991) for a standard protein recommended for a 2-5-year-old child and for adults (see Table 4.3). From the essential AA presented in the profile of the sprouts only leucine had a score below 100% (Table 4.3), being the first essential limiting AA for children and adults in Red cabbage GS, Penca cabbage GS and in Broccoli WS. In Galega kale GS and WS, leucine was also the limiting amino acid but only for children. Iqbal, Khalil, Ateeq, and Sayyar Khan (2006) found for mature kale plants lysine as the limiting AA, which was absent in the studied sprouts.

Table 4.3 Essential amino acid composition of *Brassica* sprouts (%), compared with WHO [a] "ideal protein".

Essential Amino Acids	Reference pattern			Red cabbage		Galega kale		Penca cabbage		Broccoli	
	Children	*Adults*		*Children*	*Adult*	*Children*	*Adult*	*Children*	*Adult*	*Children*	*Adult*
Isoleucine	2.8	1.3		57	122	101	214	141	301	111	237
Leucine	6.7	1.9		21	76	50	180	26	94	52	186
Phenylalanine + Tyrosine	6.3	1.9	**GS**	48	163	63	214	33	110	96	324
Threonine	3.4	0.9		312	1130	272	986	301	1091	110	400
Valine	3.5	1.3		133	346	209	546	276	721	390	1017
Limiting amino acid				leu	leu	leu		leu	leu	leu	
Isoleucine	2.8	1.3		296	630	116	246	91	195	305	650
Leucine	6.7	1.9		35	127	35	124	31	109	14	49
Phenylalanine + Tyrosine	6.3	1.9	**WS**	110	373	60	204	60	201	52	175
Threonine	3.4	0.9		262	948	395	1432	133	481	255	924
Valine	3.5	1.3		310	810	173	453	353	922	180	469
Limiting amino acid				leu		leu		leu		leu	leu

[a] WHO (1985).

Abbreviations of amino acids: leu, leucine

4.3.4. Fatty acid profile of sprouts

Gas chromatographic analysis of FAMEs in the total lipid fraction of sprouts produced under different photoperiod regimes revealed the presence of 34 different fatty acids (FA), 16 of which were saturated and 18 unsaturated (Table 4.4). The main saturated fatty acid found among all varieties of sprouts was palmitic acid (C16:0). Galega kale GS presented the highest concentration of palmitic acid, whereas the lowest amount was found in broccoli WS (see Table 4.4). This was also the main saturated FA found in leaf and seeds of kale (*Brassica oleraceae* L. var. *acpehala* DC.) (Ayaz, et al., 2006). Regarding the unsaturated FA, eicosenoic acid (C20:1) was the principal unsaturated FA in GS of Red cabbage and Galega kale followed by erucic acid (C22:1n9), linoleic acid (C18:2n6c) and oleic acid (C18:1n9c). In Penca cabbage and Broccoli sprouts the main unsaturated FAs were the erucic acid (C22:1n9), followed by eicosenoic acid (C20:1), showing a higher content of erucic acid in the sprouts germinated under darkness. In general, the amount of the main unsaturated FA was higher in WS, except for eicosenoic acid (C20:1) which was significantly lower. The presence of erudic acid was also described in the FA profile of seeds and mature leaves of kale plants (Ayaz, et al., 2006). The seeds had a higher proportion of erudic acid than the studied kale sprouts (46% in seeds *vs* 17% in GS and 36% in WS), while mature leaves had a much lower proportion of this FA (1%) in their composition (Ayaz, et al., 2006). Also the linoleic acid content found in sprouts was higher (ranging from 12% to 17%) than the levels found for leaves (12%) and seeds (12%) of kale (Ayaz, et al., 2006). The opposite was registered in relation to α-linolenic acid content, whose proportion in the sprouts (maximum of 4% in Red cabbage WS) was much lower than the one found in mature kale leaves (54%) and seeds (8%). In relation to oleic acid, sprouts presented higher amount than the leaves of mature kale plants (2%).

Photoperiod influenced significantly the profile of FAME in sprouts. From the 34 FA encountered, only capric acid (C10:0), palmitelaidic acid (C16:1n9t), cis-10-heptadecenoic Acid (C17:1) and linoleic acid (C18:2n6c) did not reveal significant ($p<0.05$) differences between sprouts growth under light/darkness cycles and only darkness. Some saturated FA like iso-tridecanoic acid (C13:0i), anteiso-tridecanoic acid (C13:0ai) and tridecanoic acid (C13:0) showed even a strong dependence on light exposures, being only present in light produced sprouts of Galega kale, Penca cabbage and Broccoli. A similar situation occurred for heneicosanoic Acid (C21:0), with exception for Penca cabbage that presented this FA

in both GS and WS. Tricosanoic acid (C23:0) exhibited the opposite behavior, appearing mainly associated to darkness condition.

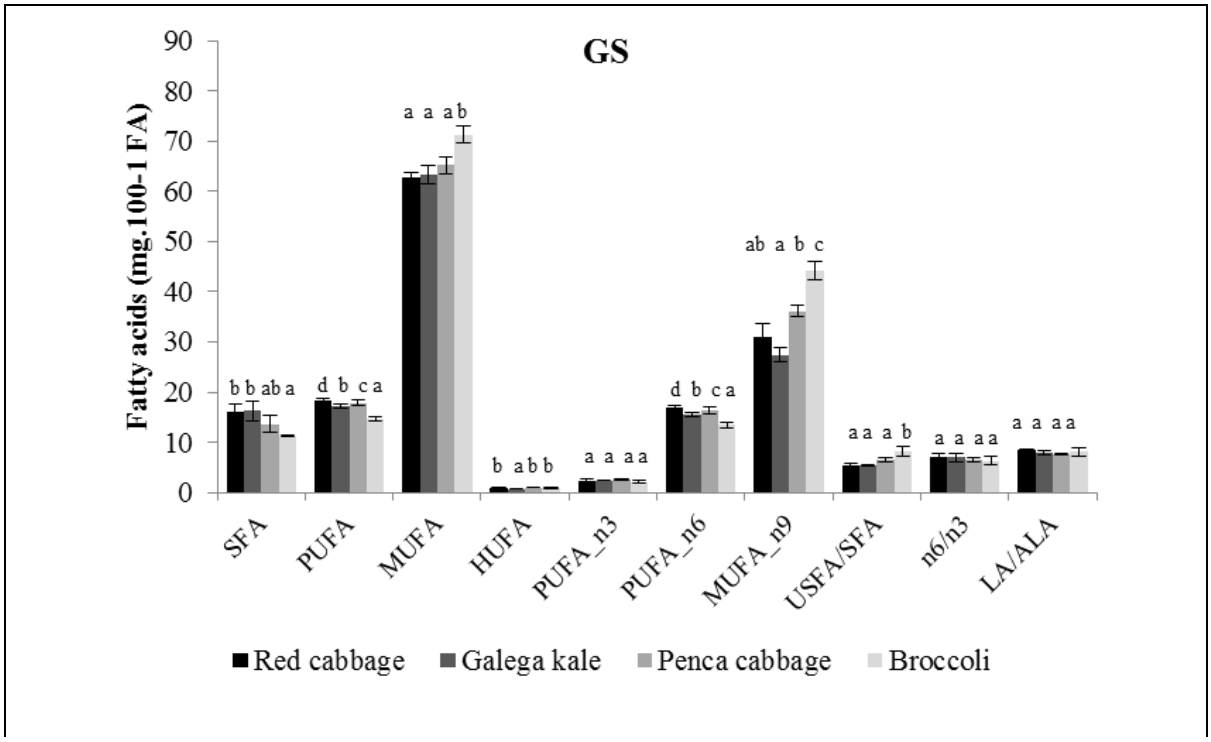
Table 4.4 Fatty acid composition of sprouts from four *Brassica oleraceae* varieties (g.100g^{-1} (dw.); mean value ± standard variation, n=3).

Fatty acids	Brassica variety								GS vs. WS (p<0.05)
	Red cabbage	Galega kale	Penca cabbage	Broccoli	Red cabbage	Galega kale	Penca cabbage	Broccoli	
	GS				WS				
C10:0 - Capric acid	0.5±0.0 a	nd	nd	0.1±0.0 b	0.1±0.0 cb	0.1±0.0 a	0.2±0.0 c	0.1±0.0 ab	ns
C12:0 - Lauric acid	0.1±0.0 ab	0.1±0.0 b	0.1±0.0 ab	0.1±0.0 a	0.1±0.0 a	0.9±0.0 b	0.1±0.0 a	0.1±0.0 a	*
C13:0i - Iso-tridecanoic acid	0.2±0.1 a	0.5±0.0 c	0.2±0.0 ab	0.3±0.1 b	nd	nd	nd	nd	*
C13:0ai - Anteiso-tridecanoic acid	0.2±0.1 bc	0.3±0.0 c	0.2±0.1 ab	0.2±0.0 a	nd	nd	nd	nd	*
C13:0 - Tridecanoic Acid	nd	0.1±0.0 c	0.1 ±0.0 a	0.1±0.0 b	nd	nd	nd	nd	*
C14:0 - Myristic acid	0.3±0.0 a	0.3±0.0 a	0.2±0.1 a	0.2±0.1a	0.2±0.1 bc	0.2±0.1 c	0.1±0.0 ab	0.1±0.0 a	*
C14:1 - Myristoleic Acid	nd	nd	nd	nd	nd	nd	0.01±0.00	nd	*
C15:0 - Pentadecanoic Acid	0.1±0.0 bc	0.1±0.0 c	0.1±0.0 ab	0.1±0.0 a	0.1±0.0 c	0.04±0.00 a	0.1±0.0 b	0.04±0.01 a	*
C15:1 - cis-10-Pentadecenoic Acid	3.0±0.2 c	2.2±0.2 b	1.5±0.0 a	1.2±0.0 a	0.04±0.00 b	nd	nd	0.03±0.00 a	*
C16:0i - Iso- palmitic acid	1.1±0.0 b	1.2±0.03 b	0.7±0.0 a	0.7±0.0 a	nd	nd	nd	nd	*
C16:0 - Palmitic acid	9.7±0.7 a	10.5±0.9 b	8.8±0.3 b	6.9±0.4 b	6.1±0.6 c	6.6±0.1 c	5.2±0.3 b	4.3±0.5 a	*
C16:1n9t - Palmitelaidic acid	0.9±0.1 a	0.9±0.1 a	1.3±0.1 a	1.0±0.1 a	0.9±0.1 b	0.9±0.1 b	0.7±0.1 a	0.8±0.1 a	ns
C16:1n7c - Palmitoleic	1.0±0.0 c	0.9±0.0 bc	0.8±0.0 ab	0.7±0.0 a	0.6±0.1 b	0.7±0.0 b	0.4±0.0 a	0.4±0.1 a	*
C17:0 - Margaric acid	0.2±0.1 b	0.2±0.1 b	0.2±0.1 ab	0.1±0.0 a	0.1±0.0 b	0.1±0.0 b	0.1±0.0 a	0.1±0.01 a	*
C17:1 - cis-10-Heptadecenoic Acid	nd	nd	nd	0.1±0.1	0.1±0.1 b	nd	nd	0.1±0.0 a	ns
C18:0 - Stearic acid	2.3±0.1 b	1.9±0.0 ab	2.0±0.1 ab	1.3±0.0 a	1.5±0.1 b	1.5±0.2 b	1.2±0.1 a	1.3±0.1 ab	*
C18:1n9t - Elaidic Acid	2.7±0.1 bc	2.9±0.1 c	2.1±0.0 ab	1.8±0.05 a	0.4±0.0 b	0.6±0.0 c	0.4±0.0 b	0.3±0.0 a	*
C18:1n9c - Oleic acid	9.8±0.8 b	6.3±0.8 a	5.7±0.0 a	8.9±0.1 b	13.4±1.2 c	10.5±0.2 b	8.7±0.0 a	10.5±0.3 b	*
C18:1n11c - Asclepic acid or *cis*-vacenic acid	1.4±0.0 bc	1.6±0.0 c	1.1±0.0 a	1.3±0.0 ab	1.3±0.1 b	1.5±0.2 b	1.0±0.1 a	1.0±0.0 a	*
C18:2n6c - Linoleic acid - LA	16.5±0.3 c	15.1±0.4 b	15.7±0.7 b	13.0±0.7 a	17.4±1.2 c	16.7±1.0 c	15.6±0.1 b	12.2±0.5 a	ns
C20:0 - Arachidic acid	0.4±0.0 b	0.3±0.0 a	0.4±0.0 b	0.4±0.0 b	0.5±0.0 d	0.4±0.0 b	0.4±0.0 c	0.4±0.0 a	*
C18:3n6 - Ɣ- linolenic acid - GLA	0.2±0.0 ab	0.2±0.0 c	0.2±0.0 b	0.2±0.0 a	0.1±0.0 a	0.1±0.0 b	0.1±0.0 a	0.1±0.0 a	*
C20:1 - Eicosenoic acid	25.2±1.0 a	30.4±1.0 b	24.4±1.42 a	22.8±1.8 a	11.4±0.4 a	15.9±0.6 d	13.7±0.2 c	13.1±0.2 b	*
C18:3n3 - α-linolenic acid - ALA	1.8±0.0 a	1.9±0.1 a	2.0±0.1 a	1.5±0.0 a	4.0±0.3 c	2.9±0.5 b	2.2±0.2 a	2.2±0.1 a	*
C21:0 - Heneicosanoic Acid	0.5±0.1 c	0.5±0.0 c	0.36±0.0 b	0.3±0.0 a	nd	nd	0.3±0.0	nd	*
C20:2 - cis-11,14-Eicosadienoic Acid	1.4±0.0 a	2.3±0.2 b	1.6±0.3 ab	1.5±0.2 ab	1.0±0.0 b	1.0±0.0 b	0.6±0.0 a	0.3±0.0 a	*
C22:0 - Behenic acid	0.5±0.0 c	0.3±0.0 a	0.4±0.0 b	0.5±0.1 bc	0.7±0.1 c	0.6±0.1 ab	0.6±0.0 b	0.6±0.0 a	*
C22:1n9 - Erucic Acid	17.7±1.4 a	17.3±1.2 a	27.1±1.6 b	32.4±1.4 b	36.7±1.2 a	35.8±1.3 a	43.9±1.3 b	48.0±1.2 c	*
C20:4n6 - Arachidonic acid - ArA	0.4±0.0 bc	0.2±0.1 a	0.4±0.1 c	0.3±0.1 ab	0.7±0.1 b	0.7±0.1 b	0.5±0.0 a	0.8±0.0 c	*
C23:0 - Tricosanoic Aci	0.01±0.00	nd	nd	nd	0.1±0.0 b	0.04±0.00 a	0.1±0.0 a	0.04±0.00 a	*
C22:2 - cis-13,16-Docosadienoic Acid	0.4±0.0 a	0.3±0.1 a	0.6±0.0 b	0.4±0.0 a	0.5±0.0 a	0.5±0.1 a	0.8±0.0 c	0.7±0.0 b	*
C24:0 - Lignoceric acid	nd	nd	nd	nd	nd	nd	0.01±0.00	nd	*
C20:5n3 - Eicosapentaenoic acid - EPA	0.6±0.1 b	0.5±0.1 a	0.6±0.1 ab	0.6±0.0 b	1.2±0.2 a	1.3±0.2 a	1.4±0.2 a	1.1±0.2 a	*
C24:1 - Tetracosenoic acid	0.9±0.0 ab	0.8±0.0 a	1.3±0.0 c	1.1±0.0 bc	1.1±0.1 a	1.4±0.1 b	1.6±0.0 c	1.7±0.1 c	*

Different letters mean significant differences (p< 0.05) between varieties within the same sprouting conditions;
***** = significant differences, ns = non-significant differences (P<0.05), between GS and WS; nd = not detected

The FA profile of sprouts revealed this products as a good source of monounsaturated FA (MUFA) (Figure 4.2), showing WS a higher proportion of MUFA. Broccoli was the variety with the higher percentage of MUFA (71.3±1.7 g.100g^{-1} FA in GS and 75.8±1.4 g.100g^{-1} FA in WS), while red cabbage had the lower MUFA content (62.7±1.0 g.100g^{-1} FA in GS and 66.0±1.4 g.100g^{-1} FA in WS). The increased of MUFA in WS was followed by a decrease of saturated FA (SFA) and an increase of highly unsaturated FA (HUFA) and polyunsaturated FA (PUFA), especially in Red cabage and Galega kale sprouts. Also MUFA n-9, PUFA n-3 and PUFA n-6 were highly represented in WS, however in the case of PUFA n-6 content in penca cabbage and broccoli, there were no statistically significant differences ($p>0.05$) between GS and WS.

The ratio of unsaturated *vs*. saturated fatty acids (Figure 4.2) showed the same tendency in the GS and WS studied, however GS had lower ratios than WS, averaging at 6.4 and 11.1, respectively. The differences between this ratios reveals a more pronounced presence of unsaturated fatty acids in WS sprouts. This different fatty acid composition could dictate a lower stability of sprouts during storage with higher amount of polyunsaturated fatty acids, as it was reported in broccoli studies (Lo Scalzo, Bianchi, Genna, & Summa, 2007). Nevertheless, in the case of sprouts, this would not be a limiting factor as these products are normally consumed in few days after harvest.



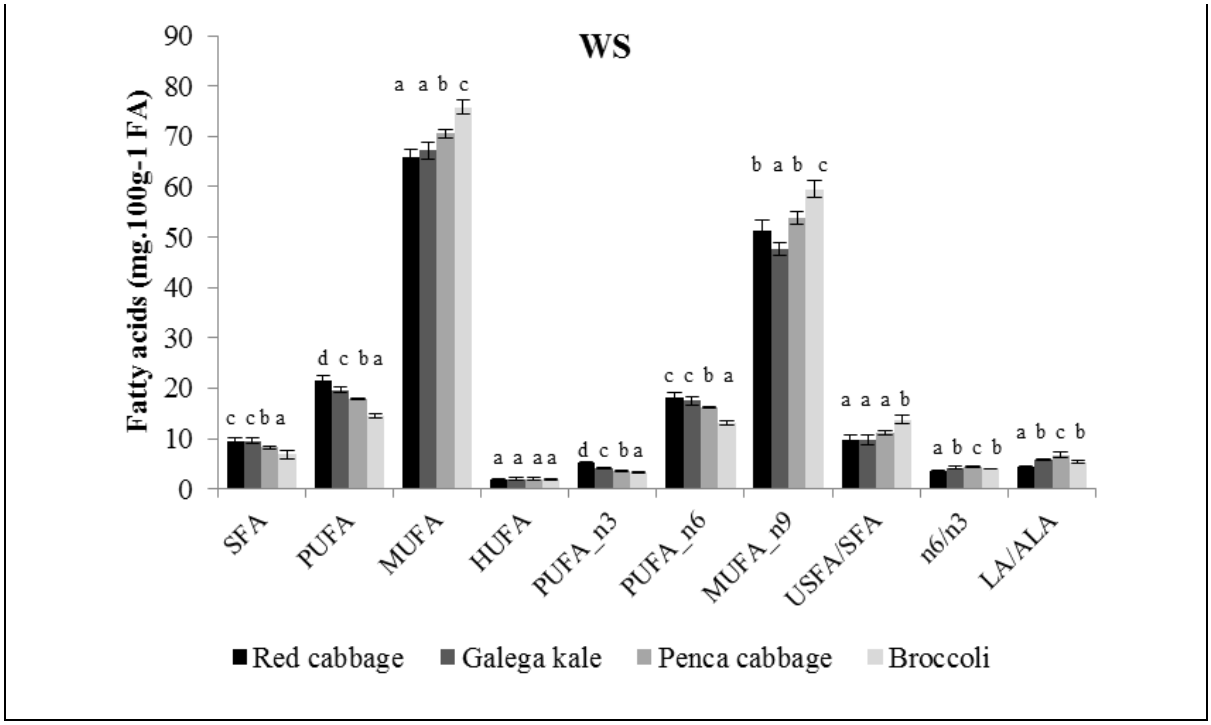


Figure 4.2 Fatty acid composition of Brassica sprouts grown under light cicles (GS) and under darkness (WS). SFA - Saturated fatty acids = ∑(C10:0, C12:0, C13:0i, C13:0ai, C13:0, C14:0, C15:0, C16:0i, C16:0,C17:0, C18:0, C20:0, C21:0, C22:0, C23:0, C24:0);

MUFA - monounsaturated fatty acids = Σ (C15:1, C16:1n9t, C16:1n7c, C17:1, C18:1n9t, C18:1n9, C18:1n11c, C20:1, C22:1n9, C24:1); PUFA - polyunsaturated fatty acids = Σ (C18:2n6c, C18:3n6, C18:3n3); HUFA - highly unsaturated fatty acids = Σ (C20:4n6,C20:5n3); PUFA n-3 = Σ (C18:3n3, 20:5n3); PUFA n-6 = Σ (C18:2n6, C18:3n6, C20:5n6); MUFA n-9 = Σ (C16:1n9t, C18:1n9t, C18:1n9c, C22:1n9); USFA/SFA = (∑C14:1, C15:1, C16:1n9t, C16:1n7, C17:1, C18:1n9t, C18:1n9c, C18:2n6c, C18:3n6, C20:1, C20:2, C20:4n6, C20:5n3, C22:1n9, C22:2, C24:1)/SFA; n6/n3 = PUFA n6/PUFA n3; LA/ALA = C18:2n6c/C18:3n3; Letters means significant differences ($p < 0.05$) between varieties for FA.

Western diets are characterized by high n-6 and low n-3 FA intake whereas in traditional diets there was a more balanced levels between n-6 and n-3 FA. The ratio n-6/n-3 found for brassica sprouts ranged from 4.0 in WS to 6.7 in GS. The recommended dietary ratio of n-6/n-3 FA for health benefits is of 1:1 to 2:1 (Simopoulos, 2008), yet the typical Western diet often contains 10 or more times the amount of n-6 relative to n-3 PUFA. WS had a lower n-6/n-3 ratio and can be considered as having healthier benefits than the GS. However, while it is accepted that PUFAs of both series are dietary essentials, the balance of n-6/n-3 fatty acids is considered somewhat controversial (Simopoulos, 2008). To this controversy contributes the difficulty to consider their intake in the context of total daily fat

and total daily PUFA consumed (i.e., as a percent of energy) and as the use of a ratio to disguise extremely low or high intakes of n-6 and/or n-3 FA; and also the fact that specific n-6 and n-3 FA represented in n-6/n-3 ratio are not always clearly defined (Deckelbaum, 2010). For both n-3 and n-6 FA, adequate intake amounts of each one are likely to be of higher utility than the use of the n-6/n-3 ratio. The predominant n-3 FA in the Western diet are the α-linolenic acid (C18:3n-3, ALA), which is common in green leafy vegetables among other foods (Simopoulos, 2009), and the linoleic acid (C18:2n-6, LA), which is the primary (in terms of mass consumed) essential FA, representing the basis of the n-6 family consumed, being present in most vegetable oils and animal meats. The ratio between the two FA LA/ALA can be used as an indicator of food quality, being a low LA/ALA ratio desired in diets as it indicates the predominance of ALA, recognized as the most potent dietary FA for reducing the total cholesterol and low-density lipoprotein (LDL-C) from the plasma (Mensink, Zock, Kester, & Katan, 2003). The suggested LA/ALA ratio are of 2:1 to 3:1 (Simopoulos, 2008). The sprouts produced under darkness had lower LA/ALA ratio, showing the Red cabbage sprouts the lowest ratio (4.3), as a result of higher ALA content, indicating that WS can be seen as healthy food product. This light exposure effect was similar to the one reported for soybean seedling (Yang, Kim, & Ha, 1982).

4.4. Conclusions

Sprouts are a low caloric food and an excellent source of a wide range of different nutrients. They also represent a new kind of ready-to-eat vegetables assigned to direct consumption in a fresh form and can also be used as important components in human healthy diets. The data provided in this study indicate that darkness conditions improved nutritional quality of sprouts, especially in what concerns their protein, dietary fiber content and, mineral and fatty acid profile. However the presence of light resulted in a higher content of selenium, whose health benefits are also recognized by the scientific community.

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CAPÍTULO 5

Evaluating the impact of environmental conditions on the glucosinolate content of *Brassica oleracea* sprouts

Phytochemistry
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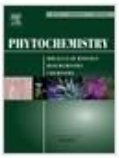

Evaluating the impact of environmental conditions on the glucosinolate content of *Brassica oleracea* sprouts

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Abstract

The glucosinolates content of brassica plants is a distinctive characteristic, representing a nutritious advantageous as many of these compounds are associated to antioxidant and anti-carcinogenic properties. Brassica sprouts are still an underutilized source of these bioactive compounds. In this work, four varieties of brassica sprouts (red cabbage, broccoli, Galega kale and Penca cabbage), including two local varieties from the North of Portugal, were grown to evaluate the glucosinolate profile and myrosinase activity during the sprouting phase. Also the influence of light/darkness exposure during sprouting on the glucosinolate content was assessed. Glucosinolate content and myrosinase activity of the sprouts was evaluated by HPLC methods. All sprouts revealed a higher content of aliphatic glucosinolates than of indole glucosinolates, contrary to the profile described for most of brassica mature plants. Galega kale sprouts had the highest glucosinolate content, predominantly sinigrin and glucoiberin, recognized by their beneficial health effects. Penca cabbage sprouts were particularly richerp in glucoraphanin, who was also one of the major compounds in broccoli sprouts. Red cabbage showed a higher content of progoitrin. Regarding myrosinase activity, Galega kale sprouts also showed the highest values, revealing that the use of light/dark cycles and a sprouting time between 7 to 9 days could be beneficial to preserve the glucosinolate content of this variety.

Keywords: glucosinolates, myrosinase activity, brassica sprouts, sprouting

5.1. Introduction

Brassica oleracea L. belongs to the *Brassicaceae* (Cruciferae) family which comprises many important vegetables, grown and consumed worldwide (Podsedek, 2007). Brassicaceae vegetables are a good source of antioxidants compounds, especially phenolics and glucosinolates (GLs) (Bruce & Pickett, 2007; Jahangir et al., 2008). In fact, only these plants and a few other edible plants from the Capparales order, are recognized as a source of all known GLs (Fahey et al., 2001).

GLs are sulfur-containing secondary plant metabolites from the β-thioglycosides group, derived from the amino acid biosynthesis (Chen & Andreasson, 2001; Podsedek, 2007). They are related to the pungent flavor and odor of Brassica vegetables (Martinez-Sanchez et al., 2006; Jones et al., 2006; Padilla et al., 2007). The GLs have a great diversity of compounds and chemical structures (Agerbirk & Olsen 2012), that diverge accordingly to species, cultivar, and even within varieties of the same species (Aires et al., 2006; Cartea et al., 2008). Besides genetic, many other factors are related to the variation of GL content in Brassica plants, namely agronomical (Aires et al., 2006), climatic (Padilla et al., 2007; Cartea et al., 2008) and environmental factors (Pereira et al., 2002; Schreiner, 2005). More than 132 individual GLs were detected and grouped into aliphatic, aromatic and indolic GLs, depending on the structure of their side-chain (Agerbirk & Olsen, 2012). From the GLs detected, 30 to 40 are present in the most economically important Brassica species (Halkier & Gershenzon, 2006).

GLs are considered health-promoting phytochemicals. The products of the enzymatic or non-enzymatic hydrolysis of GLs are biologically active compounds with diverse effects on human health (Ciska et al., 2000), including anti-carcinogenic, cholesterol-reducing, and other pharmacological effects (Cieslik et al., 2007; Verkerk et al., 2009). These substances may also act as indirect antioxidants by modulating the activity of xenobiotic metabolizing enzymes (phase I and phase II enzymes) that trigger the long lasting antioxidant activity (Vig et al., 2009), reducing the oxidative stress status responsible for triggering chronic degenerative diseases (Verkerk et al., 2009). On the other hand, intact GLs have limited biological activity (Smith et al., 2003). Their effect arises when GLs come in contact with plant myrosinase, a β-thioglucosidase or thioglucoside glucohydrolase (EC 3.2.3.1) which catalyzes the hydrolysis of GLs in Brassicas after tissue damage (Travers-Martin et al., 2008). Myrosinase is normally physically separated from the GLs in the cell, being localized in idioblasts (myrosin cells) (Andreasson et al., 2001). When plant cells are

damaged (e.g., during food processing, ingestion and digestion, or injury by predators) the enzyme is released, promoting the hydrolysis of GLs that results in a range of breakdown biologically active compounds, like indoles and isothiocyanates (Kissen et al., 2009), and also glucose and sulphate (Singh et al., 2007). Different biological activities were attributed to these compounds, some beneficial like the reduction of the risk of certain human's cancers (Fahey et al., 2001; Mithen et al., 2003), while others have a detrimental effect for humans and animals (Rosa et al., 1997). The isothiocyanates are one of the GLs breakdown products that present bio-protective effects, with anti-carcinogenic effects (Rouzaud et al., 2004), enhancing the activity of phase II enzymes and possibly inhibiting phase I enzymes (Fahey et al., 1997; Cartea et al., 2008).

Usually myrosinase stability and activity decreases during processing and domestic treatments (Oerlemans et al., 2006; Aires et al., 2012), specifically with use of heat and occurrence of cell disruption, affecting the intake and bioavailability of GLs and their breakdown products (Getahun & Chung, 1999). However food products containing active myrosinase, like Brassica sprouts and shortly cooked mature Brassica vegetables present an increased bioavailability of isothiocyanates as a result of high myrosinase activity (Verkerk et al., 2009). However, sprouts, as fresh-cut products with a short shelf life, can be susceptible to losses of GLs, due to a high myrosinase activity that can also be affected by sprouting conditions (eg. sprouting time, light exposure, harvesting and storage) (Aires et al., 2012). Sprouts are a valuable but still under-appreciated healthy dietary option which may be considered a functional food, enhancing the concentration of health-promoting bioactive compounds in the diet (Fahey et al., 1997). Besides broccoli sprouts, that showed potential anti-carcinogenic activity (Munday et al., 2008; Keum et al., 2009; Yanaka et al., 2009; Li et al., 2010), other sprouts from *Brassica oleracea* varieties are still understudied. Portuguese tronchuda cabbage and Portuguese Galega kale are traditional varieties of *B. oleracea* consumed in Portugal. The sprouts of these varieties have already demonstrated a high "in vitro" antioxidant capacity (Vale et al., 2014), which raised even more the interest in these products. The current study focuses on characterization of GLs content and myrosinase activity of Brassica sprouts produced under different light conditions and collected with different sprouting ages, in order to better define healthier sprouts and best practices of production.

5.2. Materials and methods

5.2.1. Materials and samples

All chemicals, reagents and solvents were analytical grade purchased from Sigma Chemical Co. (St. Louis, MO, USA). The water was treated with a system of thermal mantles (Isopad Isomantle, Borehamwood, Hertz, England) and in a Milli-Q water purification system (Millipore, Bedford, MA, USA).

Brassica olareacea seeds used in the study belong to the following varieties: Broccoli (*B*. *oleracea* L. var. italica Plenck, cultivar calabrese), Galega kale (*B. oleracea* var. acephala DC), Portuguese Tronchuda cabbage (*B. oleracea* L. var. costata DC, landrace Penca da Póvoa) and Red cabbage (*B. oleracea* var. capitata f. rubra). Broccoli and Red cabbage seeds were purchased at Germisem- Sementes, Lda. while seeds of Penca cabbage and Galega kale were both directly acquired from traditional farmers in the Póvoa do Varzim (Northwest of Portugal).

3.2.2. Sprouting method

Sprouting method was based on Martinez-Villaluenga et al. (2010) with slight adjustments. Seeds were sanitized for 30 min in sodium hypochlorite (0.07%, v/v), rinsed with tap water and soaked for 12 hours at room temperature, in darkness and with light agitation. Seedbed was made in polypropilene trays (10x15x4cm) containing vermiculite and seeds for green sprouts (GS) production sprouted inside a growth chamber (Fitoclima 200) with controlled temperature (25 ºC) and a photoperiod regime with cycles of 16 hours light and 8 hours darkness. White sprouts (WS) were grown at the same temperature and in darkness. Harvesting took place when sprouts reached commercial size at the 7^{th}, 9^{th} and 12^{th} days after sowing. After harvesting, sprouts were frozen at -80ºC, freeze-dried (Scanlaf model 110-4 PRO), fine-ground in a mill (Retsch ZM 200) and kept in a desiccator protected from light until analysis.

5.2.3. Glucosinolate extraction and analysis

Glucosinolates (GLs) extraction was performed according to the methodology described by Pereira et al., (2002). Briefly, 0.2 mg of freeze dried sample was extracted with 3 mL of boiling methanol 90% (v/v) and homogenised for 2 min at 24000 rpm (Utraturrax T_{25} equipped with a dispersing element S25N-10G). After 30 seconds from start boiling, 200 µL of glucotropaeolin (1 $mg.mL^{-1}$), a benzyl GL used as internal standard, was added. The homogenised sample was centrifuged for 2 min at 5000 rpm (Kubota 2100). The extraction was repeated in the residue for 1 min with 2 mL of boiling methanol 70% (v/v) and the supernatants combined to a final volume of 10 mL with methanol 70%.

Followed the purification and enzymatic desulfation of individual GLs in a small column of Sephadex DEAE A25 prepared in the laboratory (Rosa, 1978; Heaney et al., 1988). First, an aliquot of 2.5 mL of the extract was taken to dryness under air flow and resuspended in 2.5 ml of water. Meanwhile 0.5 mL of water was added to the Sephadex DEAE A25 small column and leave to drain. Then, 2x1 ml of ressuspended extract was loaded in the Sephadex DEAE A25 column in order to trap the GLs in the Sephadex DEAE A25 resin. The resin was then washed with 2x 1 ml of water followed by 2x 0.5 mL of a 0.02M piridin buffer (C_5H_5N, K22146828, Merk). Finally the adsorbed GLs were desulfated by adding 75 µl of sulfatase, prepared accordingly to Aires (2004). The reaction time was 18 hours at 20-25 ºC and after this; the small column was washed with 3x0.5 ml water, being the eluted desulfated GLs collected in glass vials and preserved at -18 ˚C until HPLC analysis. The desulfoGLs were analyzed in an HPLC system (Gilson system, HPLC 712, Gilson) using a method described by Rosa et al., (2007). The compounds were separated in a C18 column (Spherisorb 5 µm ODS2, 250 × 4.6mm i.d., Waters) and eluted with water. The mobile phase consisted of two solvents, being solvent A composed of ultra-pure water and solvent B by a solution of 20% of acetonitrile. Elution was performed at a flow rate of 1.5 $mL \cdot min^{-1}$. The chromatograms were recorded at 229 nm and GL peak identification and quantitative estimations were made using pure standard GL as internal standard (benzyl GL), and GLs response factor (Aires et al., 2012). GLs content was expressed per 100g (d.w).

5.2.4. Myrosinase Activity

The activity of the endogenous myrosinase present in sprouts was measured by the extent of hydrolysis of a known amount of sinigrin monohydrate (allyl GL), added to the incubated solution, during a short period of time (Oerlemans et al., 2006). Crude extracts from freeze dried sprouts were prepared by thoroughly mixing 500 mg of sprouts with 5 mL of ultrapure water, using a commercial blender (Ika, Ultra Turrax T_{18} basic, IKA-Labortechnik). Followed a centrifugation of the extracts at 5000 rpm for 30 min and at 4 ºC (Mettich 32R). The supernatant was collected and filtered (Whatman Nº. 1). Part of the filtrate was incubated for 1 h at 40 ºC in a water bath, to allow for myrosinase-catalyzed hydrolysis of all endogenous GLs without affecting myrosinase activity. The other part of the filtrate was incubated for 15 min at 100 ºC to inactivate the myrosinase and was used as negative control of the assay and for dilution purposes. Sinigrin (6 mM) was then added to the incubated crude extracts, in a proportion of 1 mL of sinigrin per 5.0 g of filtrate. The solution was mixed and incubated at 40 ºC for 0, 5, 10, 20, 40 and 80 min with slow agitation. The reaction was stopped by adding methanol ($9ml.mL^{-1}$crude extract) and centrifugation at 5000 g for10 min. The extracts were then treated accordingly to the method of Pérez-

Balibrea et al. (2008) with slight modifications. First, the extracts were heated at 70 ºC for 30 min, with vigorous shaking every 5min and centrifuged (17500*g*, 30 min, 4 ºC). The supernatants were evaporated under nitrogen flow, redissolved in 1 ml of ultrapure water and filtered through a 0.45 µm filter (Millipore) before injection into the HPLC system (Jasco LC-Net II/ADC) to determine the sinigrin content present in the samples. The HPLC system used comprised a pump (PU-2089 plus) with multisolvent delivery system and degasser, column oven CO-2060 Plus settled at 25 ºC, autosampler AS-2057 Plus, a C18 column (C18 YMC-Pack ODS-AQ, 5 µm and pore size 120-200 Ål, with a C18-YMC security guard, 4mm × 3mm) and a MD-2018 photodiode array detector set at 227 nm. An injection volume of 20 µL was used, with a mobile phase of 1g.L^{-1} of Trifluoro acetic acid (TFA) and an elution time of 15 min. Sinigrin content was expressed in mg per 100 g (d.w.).

5.2.5. Statistical analysis

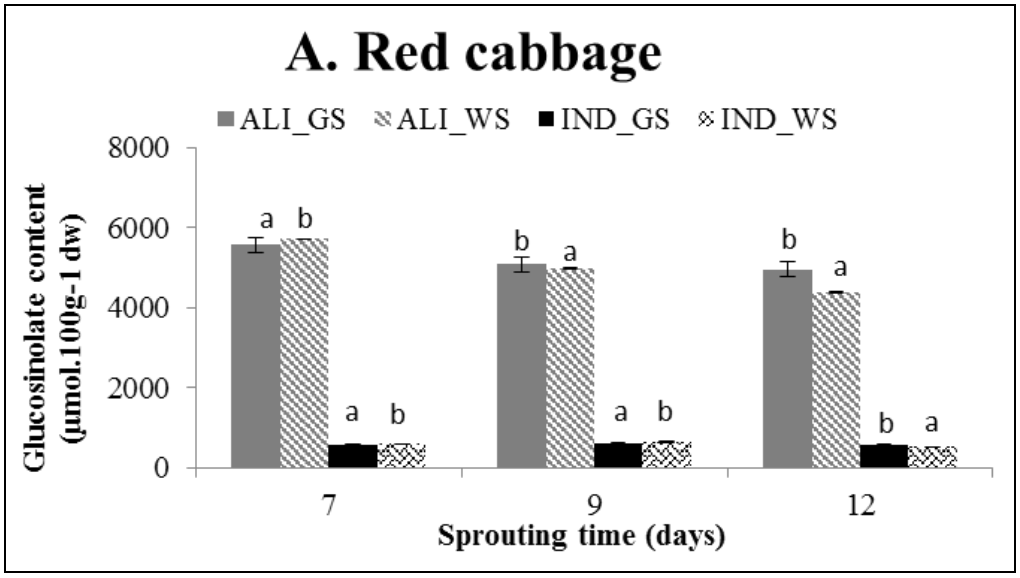
For data interpretation an analysis of variance (ANOVA and univariate) was performed using the the SPSS 20.0 software (SPSS Inc., Chicago, Illinois, EUA) for Windows. Tukey's significant difference test was used to compare means. The significance of differences was compared using the least significant difference (LSD) at 95% confidence level. Pearson's correlation coefficients (r) were determined to study the relationship between variables. Also, a linear discriminant analysis and principal component analysis were performed in order to determine the compounds that contribute most to the discriminate between the different varieties and to understand the studied factors (light exposure), respectively.

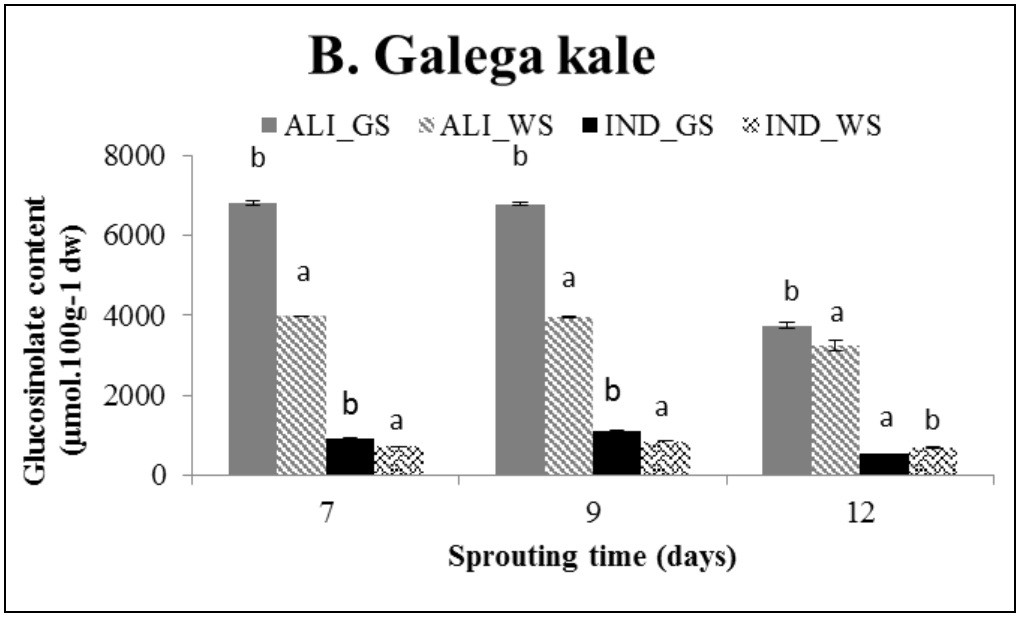
5.3. Results and discussion

5.3.1. Glucosinolates content

The GLs content of sprouts was monitored when they were ready for harvest, between the 7th and 12th day after sowing. Overall aliphatic GLs were the major GL group present in sprouts of all Brassica varieties during the three monitored stages (see Figure 1). This tendency was already observed in red cabbage and broccoli sprouts (Bellostas et al. 2007a, Baenas et al. 2012), confirming that sprouts can be a better source of aliphatic GLs than mature plants. In mature broccoli and Galega kale plants indole GLs were predominant (Aires et al. 2012) with levels of 60 and 65% of total GLs. The use of different photoperiods during sprouting had a significant effect on the production of GLs, having the sprouts produced under dark lower content of GLs (average level 4.7±0.9 mmol.100g-1 d.w.) than the produced under light/dark cycles (5.7±1.4 mmol.100g-1 d.w.). However,

some exceptions were registered, namely for red cabbage and Penca cabbage sprouts with 7 days (see Figure 5.1 A and 5.1 C). Aliphatic GLs were the major GLs group in sprouts with higher expression in GS (88%), whereas WS had an average level of aliphatic GLs of 4.0±0.9 mmol.100g-1 d.w., which represented 84% of the total GL content. The opposite was seen regarding the influence of the photoperiod on the indole-GL content, as the absence of light led to a significant effect on these GLs content, having most of the samples grown under darkness a higher content of Indole-GL (see Figure 5.1A, 5.1C and 5.1D), with exception of Galega kale sprouts in which levels were similar between GS and WS.





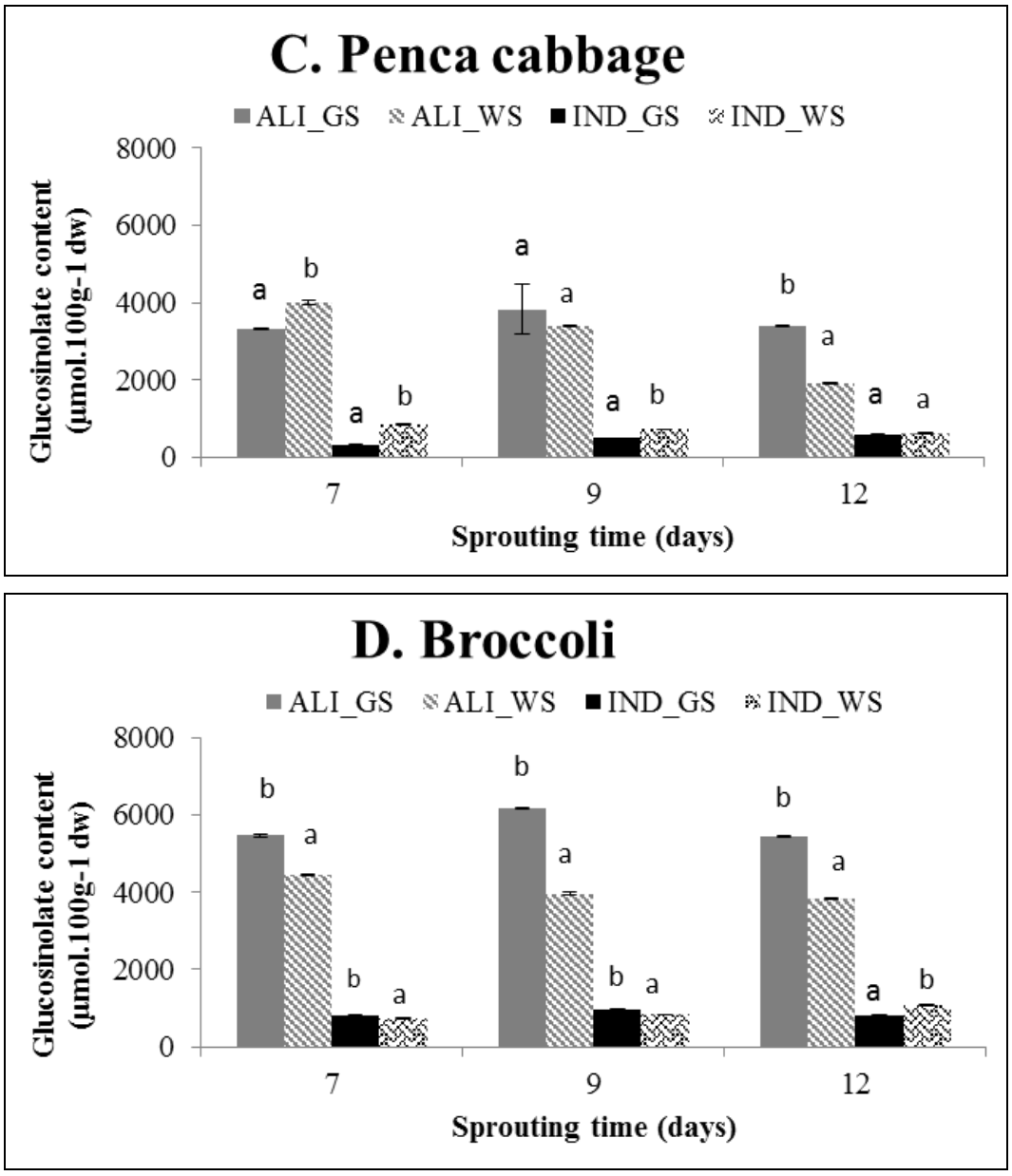


Figure 5.1 Total Aliphatic (ALI) and total indole glucosinolates (IND) of four Brassica sprouts at 3 different spouting times, under light (GS) and darkness (WS) conditions. Differences between WS and GS with the same letters are non-significant ($p < 0.05$).

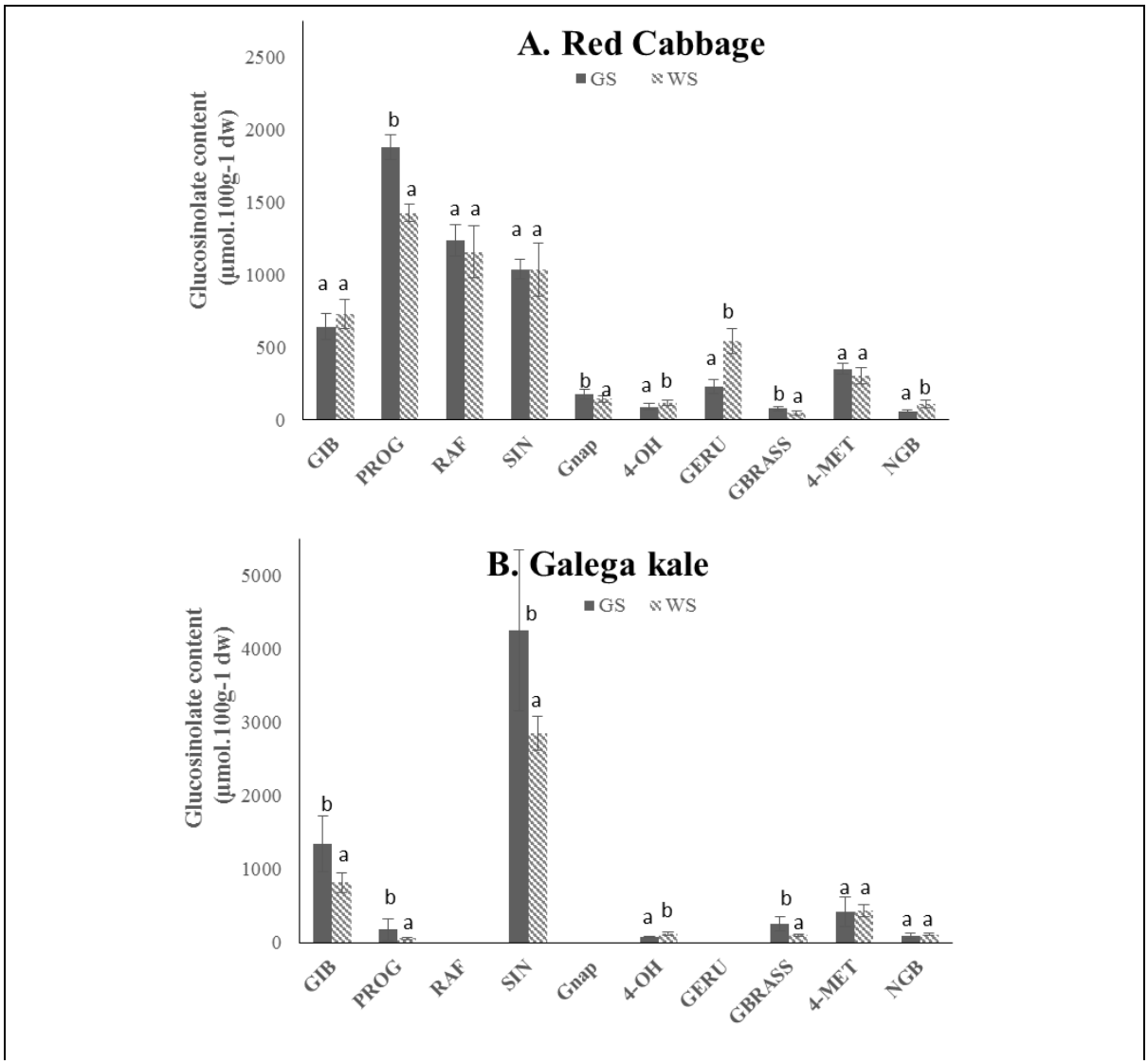
Galega kale produced under light conditions showed the highest aliphatic GLs content (5.8±1.8 mmol.100g-1 d.w.) followed by broccoli (5.7±0.4 mmol.100g-1 d.w.), red cabbage (5.2±0.3 mmol.100g-1 d.w.) and Penca cabbage (3.5±0.2 mmol.100g-1 d.w.). Aliphatic GLs content found in red cabbage GS were also higher than the values found in mature plants (3.8 mmol.100g-1 d.w.) reported by Volden et al. (2008). Under dark conditions the total aliphatic GLs content rank changed, being as follows: red cabbage >

broccoli > Galega kale > Penca cabbage. Aliphatic GLs content under dark conditions was lower than under light in three of the studied varieties, corresponding to 83% in Galega kale (vs 87% in GS), 80% in Penca cabbage (vs 88% in GS) and 82% in broccoli (vs 86% in GS), whilst on red cabbage represented 89% for both GS and WS. The lowest content of indole GLs (0.5±0.1 mmol.100g-1 d.w.) were registered on Penca cabbage GS, whilst sprouts from broccoli were the better source of indole GL (average content of 0.9±0.2 mmol.100g-1 d.w. for both GS and WS), followed by Galega kale GS and WS (0.8±0.1 mmol.100g-1 d.w.), Penca cabbage WS (0.7±0.05 mmol.100g-1 d.w.) and red cabbage WS and GS (0.6 mmol.100g-1 dw).

The time of sprouting was also an important factor to benefit from higher concentrations of aliphatic GL. Longer sprouting times tend to decrease aliphatic GLs concentration, especially after 12 days of sprouting. Overall sprouts with 7 days of germination had a significantly ($p< 0.05$) higher content of total and of the aliphatic GLs (see Figure 5.1). However, in relation to indole GLs content, the highest values were reached for sprouts grown during 9 days. Some exceptions were registered, namely in Galega kale sprouts, where no differences were detected between 7th and 9th day of sprouting and between GS and WS aliphatic GLs content. Another exception was seen in broccoli sprouts GLs content, where GS with 9 days had a higher aliphatic GLs content, and WS with 12 days showed a higher indole GLs content. The decreasing of GL total level in sprouts with longer sprouting period was also recorded for red cabbage and broccoli by Baenas et al. (2012).

The composition of glucosinolates profile is important as the beneficial effects resulting from the presence of glucosinolates depend on the nature of the breakdown products, after degradation and absorption. The GLs profile of the four brassica varieties is presented in Figure 5.2. Regarding the individual aliphatic GLs of all the sprouts studied, sinigrin was highest in GS (7.4±1.1 mmol.100g-1 dw), followed by glucoraphanin (4.6±1.3 mmol.100g-1 d.w.) and glucoiberin (3.8±0.3 mmol.100g-1 d.w.). Sprouts produced under dark conditions showed a 21% reduction in sinigrin level, 22% in glucoraphanin and 8% in glucoiberin. The GLs profile of sprouts was an intermediate between the one found in seeds and the described for mature tissues (Bellostas et al., 2007b; Brown et al. 2003), which explains the predominance of aliphatic GL rather than the indole GL found by several researchers in mature vegetables (Fahey et al., 1997; Brown et al., 2003; Volden et al., 2008; Aires et al., 2012). The predominance of indole GL in mature plants was related to “de novo synthesis” of this group of GL during growth (Chen & Andreasson, 2001). These differences increase the nutritional importance of the sprouts, particularly Galega kale GS

and Penca Cabbage, both with a high content of sinigrin, an aliphatic-GL, that together with glucoraphanin (only in Penca cabbage) are known as an important source of isothiocyanates, a potent inducers of phase II enzymes, with proved action in cancer prevention (Fahey et al., 1997; Barillari et al., 2005). In individual indole-GLs the highest concentration was observed in WS with 1.7±0.1 mmol.100g-1 d.w. of 4-methoxyglucobrasscin followed by 0.5±0.02 mmol.100g-1 d.w. of neoglucobrassicin and 0.5±0.02 mmol.100g-1 d.w. of 4-hydroxyglucobrassicin. Individually, red cabbage sprouts showed a greater diversity in GLs profile, containing ten different GLs. Progoitrin, glucoraphanin and sinigrin represented the major GLs present in Red cabbage, accounting for 33%, 21% and 18% of total GLs in GS and 25%, 21% and 19% in WS. Under light conditions sprouts were significantly richer in progoitrin than the produced under darkness. Progoitrin is considered an antinutrient that can cause goitrogenic effects, and whose presence as one of the major GLs of red cabbage sprouts and mature plants was also reported by Baenas et al. (2012) and Ciska et al. (2000), respectivaly. Glucoraphanin, sinigrin and glucobrassincin were also described as major GLs in the profile of red cabbage mature plants (Meyer & Adam, 2008). The levels of the indole glucobrassicin were significantly lower in sprouts, representing only 1.4% of the total GLs content in GS and 0.8% in WS. Gluconapin, an aliphatic GLs, was exclusive of red cabbage sprouts, but the mean concentration encountered (0.16± 0.03 mmol.100g-1 d.w.) was lower than the levels found in mature plants (Meyer & Adam, 2008; Volden et al., 2008). The same happened in the 4-methoxyglucobrassicin content, described as a major indole GLs of red cabbage mature plants (Volden et al., 2008), but that only represented 6% of total GLs in red cabbage sprouts.


A. Red Cabbage
GS
WS
Glucosinolate content (µmol.100g-1 dw)
2500
2000
1500
1000
500
0
GIB
PROG
RAF
SIN
Gnap
4-OH
GERU
GBRASS
4-MET
NGB
B. Galega kale
GS
WS
Glucosinolate content (µmol.100g-1 dw)
5000
4000
3000
2000
1000
0
GIB
PROG
RAF
SIN
Gnap
4-OH
GERU
GBRASS
4-MET
NGB

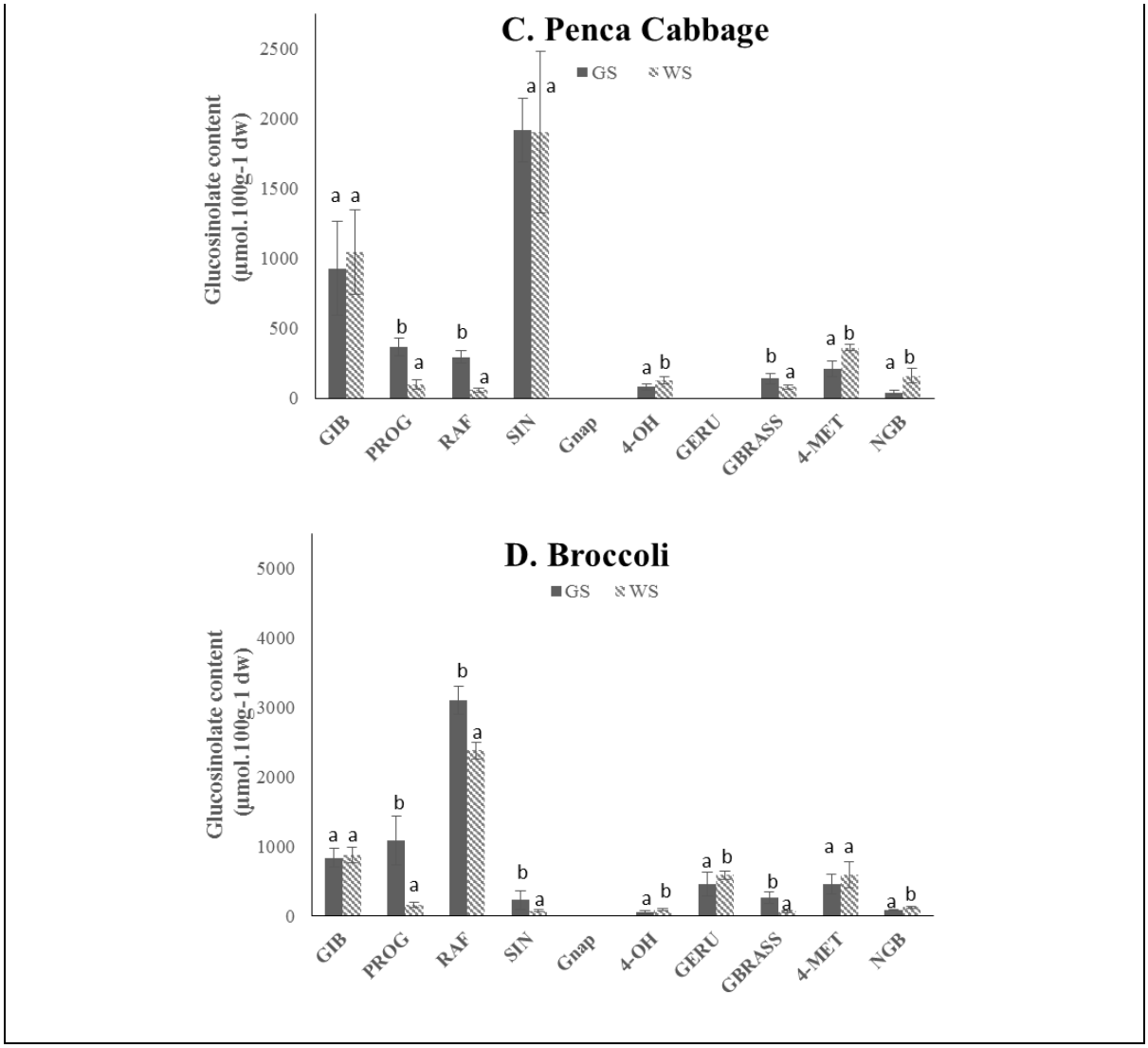


Figure 5.2 Glucosinolate profile identified in sprouts of red cabbage, Galega kale, Penca cabbage and broccoli betwwen the 7th and 12th day of sprouting (mean value ± standard deviation, n=9, expressed in µmol.100g-1 d.w.; differences between white sprouts (WS) and green sprouts (GS) with the same letters are non-significant ($p < 0.05$)). Abbreviations: GIB, glucoiberin; PROG, progoitrin; RAF, Glucoraphanin; SIN, sinigrin; Gnap, gluconapin; 4-OH, 4-hydroxyglucobrassicin; GERU, glucoerucin; GBRASS, Glucobrassicin; 4-MET, 4-methoxyglucobrassicin; NGB, neoglucobrassicin.

The Portuguese varieties, Galega kale and Penca cabbage, presented a similar GL profile, with exception for glucoraphanin that was not detected in Galega kale sprouts. Glucoraphanin is considered an important and desirable GL since sulphoraphane, the isothiocyanate (ITC) from glucoraphanin, is considered the most potent inducer of phase II enzymes (Fahey & Talalay, 1999; Bellostas et al., 2007a), representing one of the nutritional

advantages of Penca cabbage sprouts. The Penca cabbage GS showed also a significantly ($p<0.05$) higher glucoraphanin content (0.30± 0.04 mmol.100g-1 d.w.) than the sprouts produced under darkness (0.06± 0.01mmol.100g-1 d.w.). The major GLs of the studied Portuguese varieties were sinigrin and glucoiberin, whose degradation products are prop-2-enyl ITC and iberin, respectively. These compounds are also inducers of phase II enzymes and have antiproliferative activity (Wallig et al., 1998; Canistro et al., 2004). Sinigrin and glucoiberin accounted respectively for 64% and 19% of the total GLs in Galega kale and 48% and 25% in Penca cabbage. The third most abundant GLs in these sprouts was 4-methoxyglucobrassicin, having the WS a significantly higher ($p< 0.05$) content than GS and accounting for 9% of the total GLs. The 4-methoxyglucobrassicin levels found represented less 30% than the proportion in Portuguese cabbage mature plants (Aires et al., 2012), nevertheless the content was higher than the related for cabbage sprouts (Kestwal et al., 2011).

The glucosinolate profile of broccoli GS sprouts showed glucoraphanin as the most prominent GL in both GS and WS broccoli sprouts (represented 48% of total GLs), which is in agreement with the results of most broccoli sprouts varieties (Charron et al., 2005; Tian et al., 2005). Broccoli sprouts are normally considered a better source of glucoraphanin than mature plants (Meyer & Adam, 2008), that showed as the most bioactive isothiocyanates, the sulforaphane (derived from glucoraphanin), the allyl isothiocyanate (derived from sinigrin) and indole-3-carbinol (derived from glucobrassicin) (Jones et al. 2006). In broccoli sprouts, in addition to glucoraphanin, the progoitrin (17%) and glucoiberin (13%) in GS, and glucoiberin (18%) and the 4-methoxyglucobrassicin (12%) in WS, were the major GLs presented. The absence light during sprouting had a clear effect on the GLs profile of broccoli sprouts, causing a 13% decreased in the progoitrin levels in WS. That effect was also seen in sinigrin levels, were it represented 4% to the total GLs in GS and 2% in WS. The sprouts produced under darkness were significantly richer in glucoerucin and 4-methoxyglucobrasscin (more 5%) than the GS.

The discriminant analysis with the method Wilk's Lambda was used to identify the GLs that allow to significantly discriminate the varieties (Figure 5.3). The discriminant analysis extracted two discriminant functions retaining as statistically significant and highly tolerant (>0.8) the GLs gluconapin, glucoraphanin and sinigrin. Function 1 and function 2 explain 52% and 47%, respectively. The canonical correlations between the discriminant functions and the GLs were of 0.97 for the function 1 and 0.96 for the function 2. This analysis allowed to clearly distinguish between the broccoli and red cabbage sprouts from

the other two varieties, Penca cabbage and Galega kale. These latter, were grouped together due to their similar GLs composition.

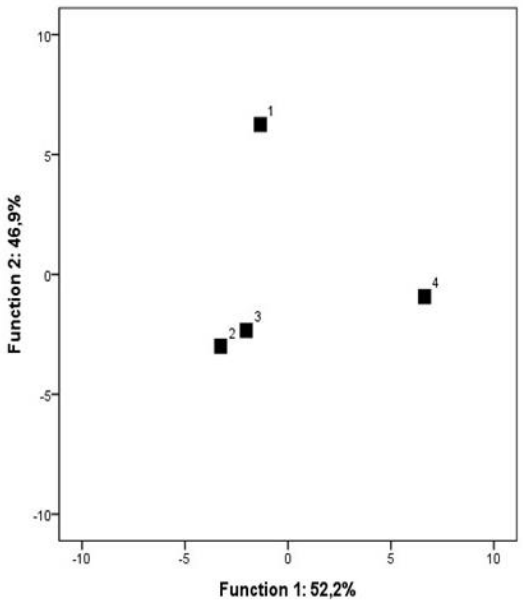
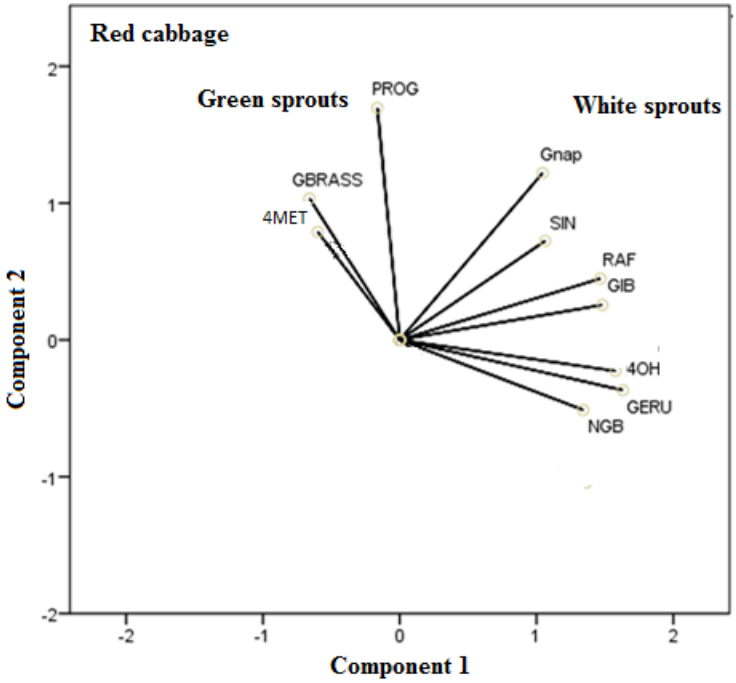


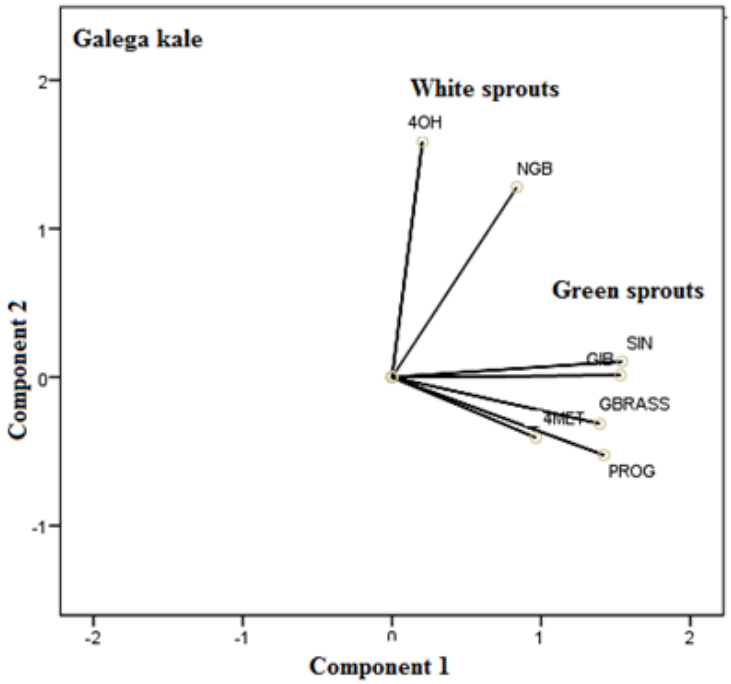
Figure 5.3 Discriminant functions extracted from the discriminant analysis. 1= red cabbage; 2 = Galega Kale; 3 = Penca cabbage; 4 = Broccoli.

The principal component analysis (PCA) was applied to each variety in order to better understand the influence of light exposure during sprouting in GLs composition (Figure 5.4). These analysis allowed for the representation of GS and WS in two different components, explaining more than 80% of the total variance of the original variables. In red cabbage, broccoli and Penca cabbage, GS are mainly represented by component 2, being progoitrin, glucobrassicin and glucoraphanin (for broccoli and Penca cabbage) and 4-methoxyglucobrassicin (for red cabbage) the main differentiating GLs between GS and WS. Galega kale GLs results presented a different behavior, being the component 2 represented mainly GS, with 4-hydroxyglucobrassicin and neoglucobrassicin as the main GLs responsible for the differentiation between GS and WS (intensity weight of 0.97 and 0.78, respectively). The component 1 showed always to be more consistent than the component 2, revealed by their higher Cronbach's Alpha, whose value ranged from 0.86 in Galega kale and 0.93 in Penca cabbage. Nevertheless the consistency of the component 2 was always higher than 0.5, showing a moderate consistency considering that it has always associated less GLs than the component 1.

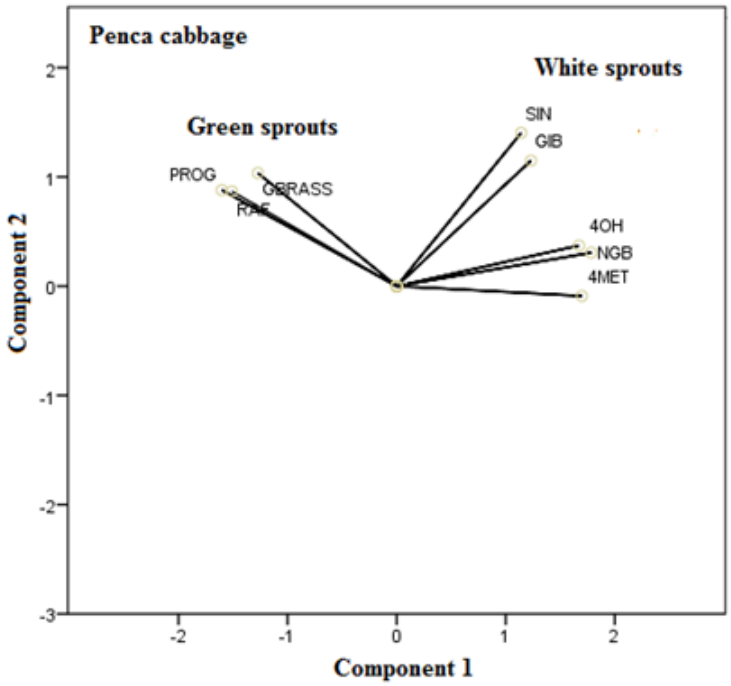
A -


Red cabbage
Green sprouts
White sprouts
PROG
Gnap
GBRASS
4MET
SIN
RAF
GIB
4OH
GERU
NGB
Component 2
Component 1
2
1
0
-1
-2
-2
-1
0
1
2

B –


Galega kale
White sprouts
4OH
NGB
Green sprouts
SIN
GIB
GBRASS
4MET
PROG
Component 2
Component 1
2
1
0
-1
-2
-1
0
1
2

C -



D –

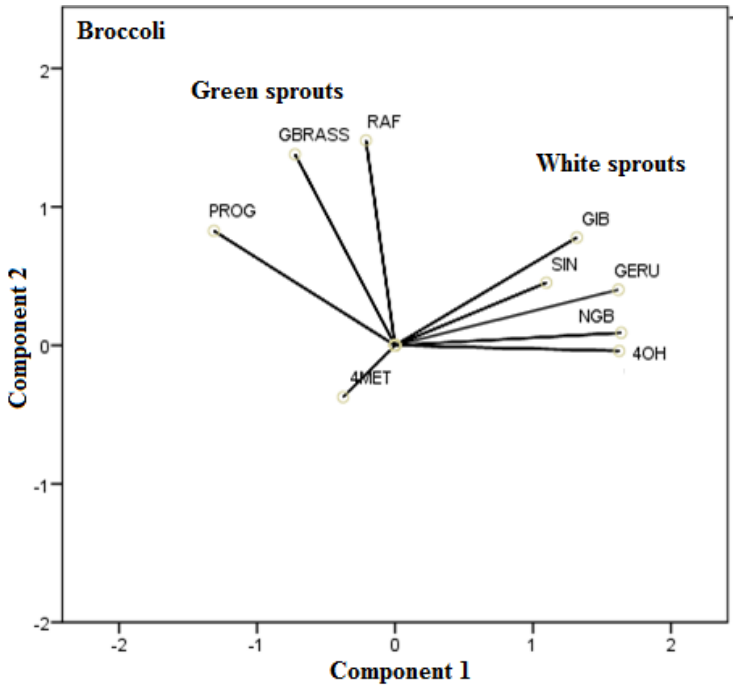


Figure 5.4 Principal component analysis (PCA) results, aggregating glucosinolates in white sprouts (WS) and green sprouts (GS). (For abbreviations see figure 5.2 legend).

5.3.2 Myrosinase activity

The results regarding the activity of myronase activity on brassica sprouts are presented in Figures 5.5 and 5.6. Myrosinase activity of sprouts was monitored for 80 min, however no sinigrin was detected after 40 min of incubation, revealing that was completely hydrolyzed by myrosinase (see Figure 5.5). Broccoli WS showed the most intense myrosinase activity, whilst red cabbage showed the lowest myrosinase activity, despite some enzymatic activity after 40 min of incubation. In broccoli sprouts almost all sinigrin was hydrolyzed after 5 min incubation in WS when compared to the results obtained in the other varieties (see Figure 5.5D). Galega kale and Penca cabbage sprouts were similar ($P>0.05$) on its capacity to breakdown sinigrin showing enzymatic activity in most GS until 10 min of incubation (Figure 5.5B and 5.5C). Glucosinolates are mostly hydrolyzed by plant myrosinase in the small intestine and in the mouth, playing an essential role in the conversion of glucosinolates in humans. The inactivation of plant myrosinase will consequently affect the dietary absorption of bioactive compounds, therefore the preservation of plant myrosinase intact as much as possible represents a health advantages that comes from the consumption of *Brassica* vegetables (Aires et al., 2012). *Brassica* plants with high myrosinase activity can exhibit a high rate of natural GLs losses (Aires et al., 2012), thus sprouting conditions and handling should be optimize to improve the potential nutritional benefits of sprouts.

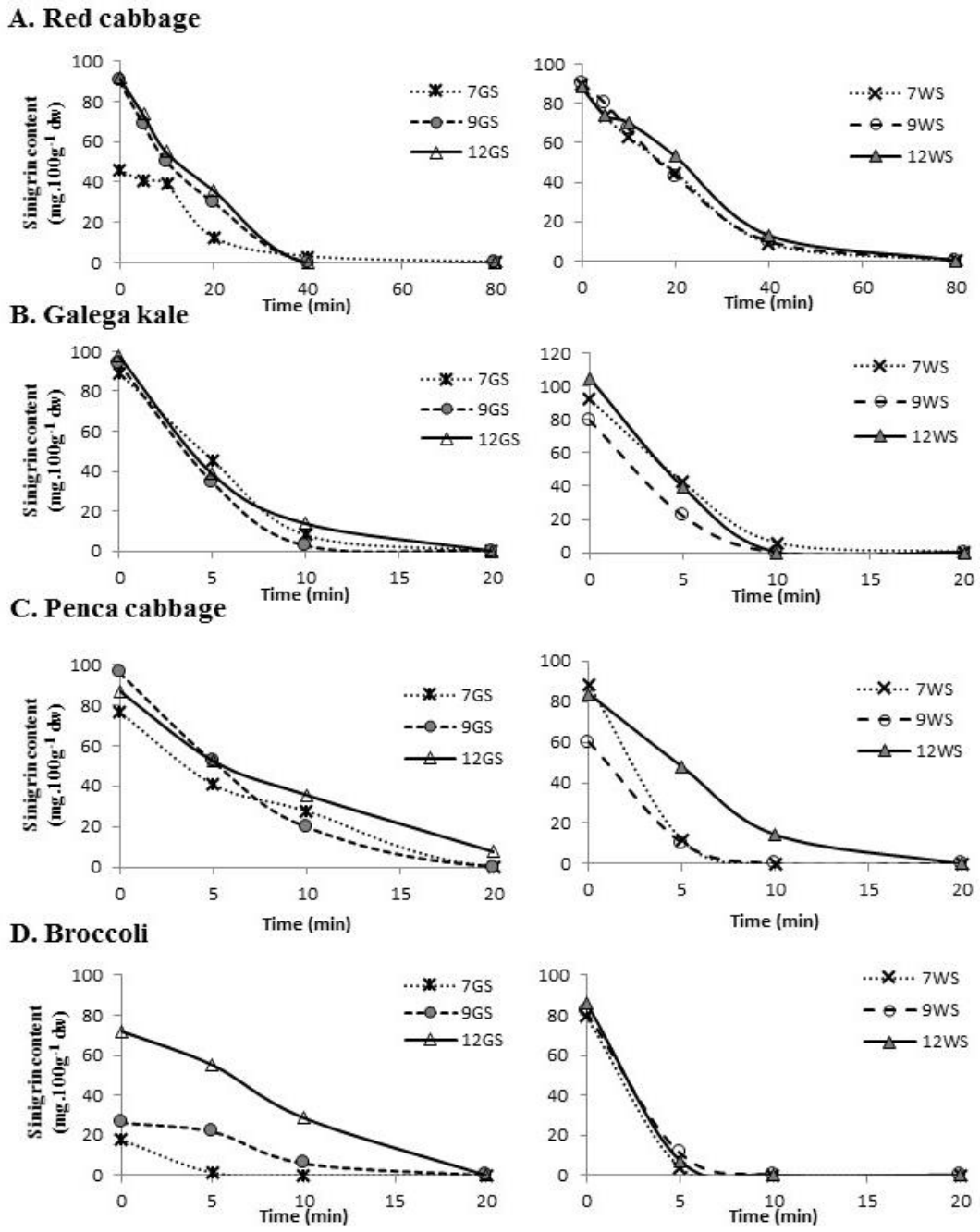
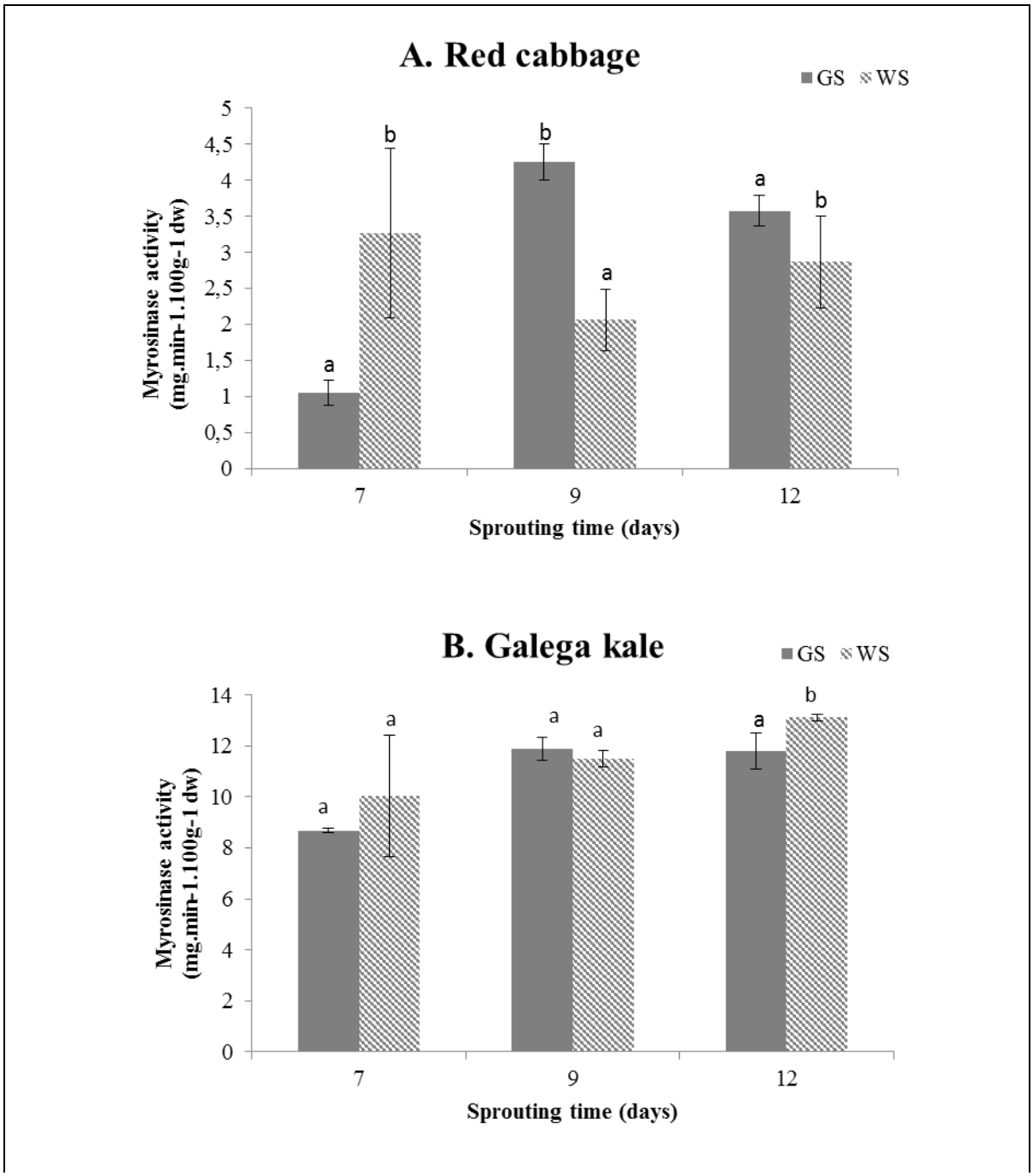


Figure 5.5 Residual evolution of exogenous sinigrin during a maximum time of 80 min in red cabbage sprouts and 20 min in the other *Brassica* varieties. Sprouts with different sprouting times and different photoperiod were analyzed: 7GS – 7 days sprouting time under light, 7WS – 7 days sprouting time under darkness, 9GS – 9 days sprouting time under light, 9WS – 9 days sprouting time under darkness, 12GS – 12 days sprouting time under light, 12WS – 12 days sprouting time under darkness.

In order to better understand the behavior of sprouts capacity to breakdown sinigrin, the lower common incubation time (5 min) was selected to evaluate the activity of myrosinase per minute (mg.min-1.100g-1 dw) (Figure 5.6). Most of the sprouts produced under darkness showed an intense myrosinase activity, with no exogenous sinigrin detected after 5 min incubation (Figure 5.5). Exceptions were registered in Red cabbage, Galega kale with 7 days sprouting and Penca cabbage with 12 days. In general, the presence of light reduced myrosinase activity since at 5 min incubation it was possible to verify that GS samples presented higher level of sinigrin as a result of lower myrosinase activity (see Figure 6A, C and D). Besides light exposure, the sprouting time was also determinant for the capacity of sprouts to hydrolyze sinigrin. Young sprouts were more susceptible to losses of GLs since sprouts with 7 days showed lower levels of exogenous sinigrin, whilst the GLs degradation capacity tends to be reduced with sprouting time. Although it was observed that the majority of sprouts produced under darkness had higher myrosinase activity, red cabbage WS with 9 days sprouting where an exception. Broccoli GS were the most affected by light exposure showing an enzymatic activity 83% lower than the registered in WS (15 $mg.min^{-1}.100g.^{-1}$ dw) which may indicate also a higher rate of natural degradation of GLs in sprouts produced under dark conditions (Figure 5.6D). Galega kale sprouts showed similar myrosinase activity between GS and WS, until the 12 days of sprouting, when WS revealed a higher activity. Penca cabbage revealed the opposite behavior, with the myrosinase activity decreasing over the sprouting time, especially in WS. Sprouts are normally consumed in a fresh state, preserving the myrosinase activity. In this way, sprouts harvest with a lower myrosinase activity can preserve more their GL content. The 7 days sprouts under light can benefit from lower myrosinase activity in all varieties, except in broccoli since the lowest activity of the enzyme was found at 9 days sprouting (see Figure 5.6). Nevertheless the myrosinase activity in broccoli under light was always very low relatively to the other varieties. Penca cabbage showed an intermediate myrosinase activity, being higher than in red cabbage sprouts and lower than in Galega kale sprouts.

Pearson's correlation between myrosinase activity and GLs was analyzed and absolute values are presented in Table 5.1. All the GLs were correlated with myrosinase activity except the indole GLs, 4-methoxyglucobrassicin and glucobrassicin. A very strong correlation was found for progoitrin and a strong one for gluconapin. The other GLs content was only moderately correlated with myrosinase activity. These results may suggest that myrosinase coexisted with glucosinolates in Brassica sprouts; however, there is no clear relationship between the myrosinase activity and all GLs found, since the pearson correlations were mainly moderated.


A. Red cabbage
GS
WS
Myrosinase activity (mg.min-1.100g-1 dw)
5
4,5
4
3,5
3
2,5
2
1,5
1
0,5
0
a
b
b
a
a
b
7
9
12
Sprouting time (days)
B. Galega kale
GS
WS
Myrosinase activity (mg.min-1.100g-1 dw)
14
12
10
8
6
4
2
0
a
a
a
a
a
b
7
9
12
Sprouting time (days)

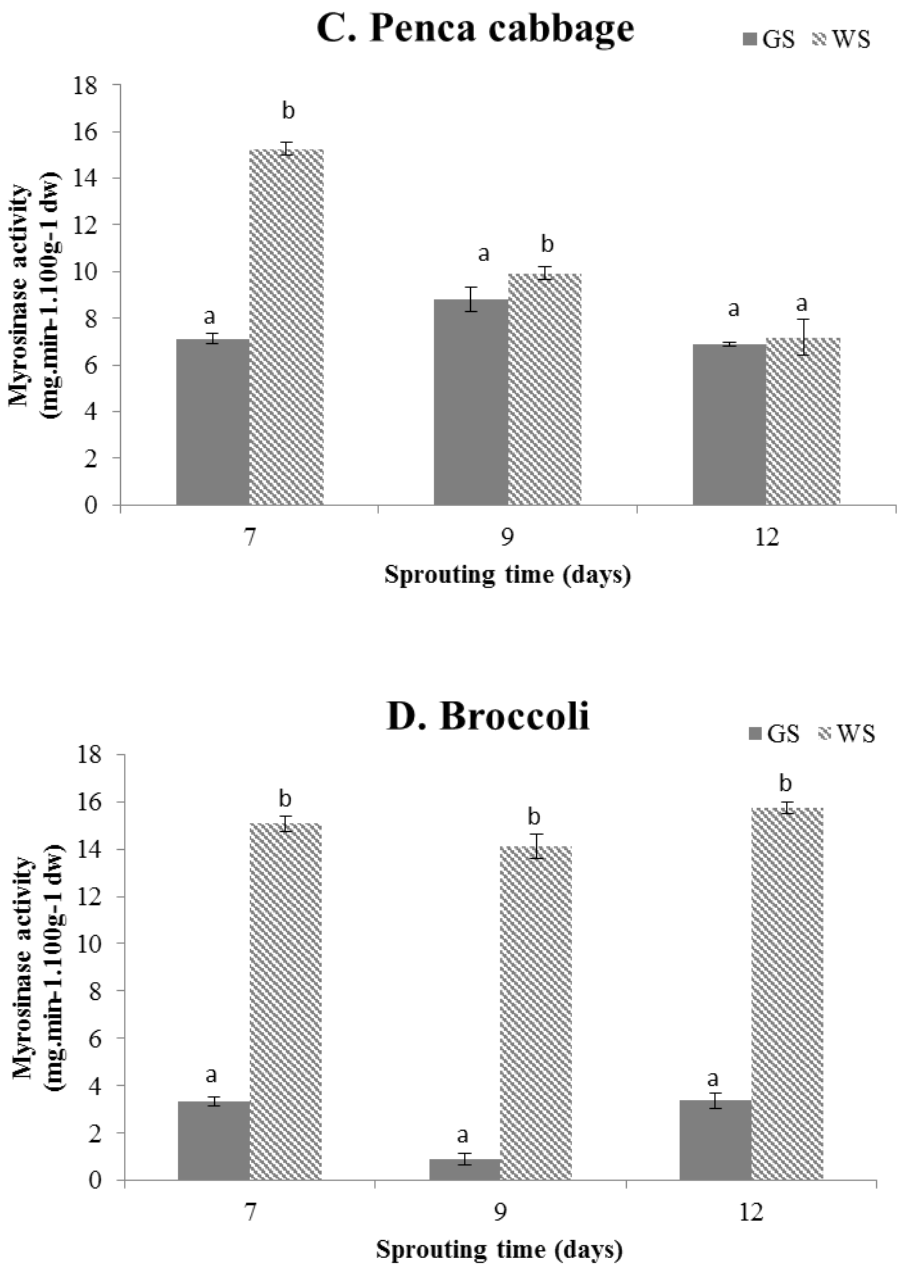


Figure 5.6 Myrosinase activity (mg.min-1.100g-1 d.w.), based on the residual evolution of exogenous sinigrin between zero and five minutes, in sprouts of four *Brassica* varieties. Abbreviations: GS – green spouts, WS – white sprouts. Differences between WS and GS with the same letters are non-significant ($p < 0.05$).

Table 5.1 Pearson's correlations between myrosinase activity and glucosinolates.

	Pearson Correlation - Myrosinase activity ($mg.min^{-1}.100g^{-1}$ dw)	*Sig.*
Glucoiberin	0.380**	0.001
Progoitrin	0.809**	0.000
Glucoraphanin	0.301*	0.01
Sinigrin	0.299*	0.011
Gluconapin	0.619**	0.000
4-Hydroxyglucobrassicin	0.230	0.052
Glucoerucin	0.258*	0.029
Glucobrassicin	0.115	0.336
4-Methoxyglucobrassicin	0.348**	0.003
Neoglucobrassicin	0.441**	0.000
Total GLs	0.314**	0.007
Aliphatic GLs	0.394**	0.001
Indole GLs	0.373**	0.001

** Correlation is significant at the 0.01 level
* Correlation is significant at the 0.05 level

5.4. Conclusions

The glucosinolate content of brassica vegetables is recognized as one of their main nutritional advantages, gaining even more relevance in sprouts as they are consumed in a raw state, preserving their natural glucosinolate content. Within the studied sprouts varieties Galega kale stood out by their higher glucosinolate content, especially when sprouted under light and darkness cycles. Galega kale is a traditionally consumed brassica plant in Portugal and its sprouts showed to be an important source of aliphatic GLs. The consumption of Galega kale sprouts, with sprouting times between 7 and 9 days, can be a healthy component of the diet able to supply inducers of phase II enzymes. The preservation of these autochthonous varieties can be made by diversifying gastronomic recipes with introduction of sprouts as a healthy food and as an alternative to typical Galega kale. However, a special attention on handling sprouts is necessary since plant myrosinase should be kept intact as much as possible to optimize their health benefits. Myrosinase activity in Galega kale sprouts could be a drawback as it was high when compared with the other varieties, only exceed by the one found in broccoli sprouts grown under dark conditions. Thus sprouting under light/darkness cycles with shorter sprouting phases is recommended to promote a higher GL content, but it also requires that harvesting and

handling until consumption must be performed with care to avoid GLs degradation due to the higher myrosinase activity of these sprouts. Sprouts produced under darknes, besides the lower GL content showed a higher myrosinase activity that could compromise the GL content in the moment of consumption due to the necessary handling procedures.

Acknowledgements

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CAPÍTULO 6

Phytochemical composition and Antimicrobial properties of four varieties of *Brassica oleracea* sprouts

Food Control
Submited

Phytochemical composition and Antimicrobial properties of four varieties of *Brassica oleracea* sprouts

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Abstract

Brassica sprouts are within the most common and most widespread sprout varieties in the market, attracting consumers looking for a healthy and convenient food source of beneficial phytochemicals. In this study, four different varieties of *Brassica oleracea* sprouts (red cabbage, broccoli, Galega kale and Penca cabbage) were grown under different light exposure conditions and during different periods of germination to investigate the influence of these factors on their phytochemical composition. The presence of phenolic compounds and the organic acids profile of each variety was analyzed by HPLC-DAD methods, being also studied the potential antimicrobial activity of the brassica sprouts and how that could be correlated to their phytochemical composition. All samples revealed a predominance of hydroxycinnamic acids in their phenolic profile that ranged from 213 µg.g $^{-1}$ in broccoli to 252µg.g $^{-1}$(dry weight basis) in Penca cabbage sprouts. Regarding their organic acids determination, each variety showed a characteristic profile, sharing a predominance of citric (67%), malic (19%) and oxalic (13%) acids in their composition. The organic acids content was also significantly ($p<0.05$) influenced by the sprouting light conditions and duration. All sprout extracts revealed a potential antimicrobial activity of these brassica varieties against some of the most challenging foodborne pathogens. The best antimicrobial activities were found in the red cabbage and broccoli extracts, showing also a strong correlation with the organic acids found in the sprouts composition.

Keywords: brassica sprouts, phenolic compounds, organic acids, antimicrobial activity, sprouting conditions.

6.1. Introduction

The consumption of sprouts is increasing at a worldwide level due to their widespread availability and high nutrient content (Yang et al., 2013). Sprouts result from a process of seed germination and are presented as one valuable alternative increase the consumption of different seeds in human nutrition. The use of seed sprouts as food has spread from Far Eastern countries to the Western world and consumers can find on the market a wide variety of different types of sprouts, in which the Brassicaceae family is well represented. Sprouts from some specific Brassicaceae plants have been studied, particularly their sensory quality in terms of consumers' acceptance (Troszyńska, Lamparski & Kozłowska, 2002), their antioxidant capacity (Martinez-Villaluenga et al., 2010; Oh & Rajashekar, 2009; Podsędek, 2007; Vale, Cidade, Pinto & Oliveira, 2014) and their composition in bioactive phytochemicals (Fahey, Zhang & Talalay, 1997; Moreno, Pérez-Balibrea, Ferreres, Gil-Izquierdo, & García-Viguera, 2010; Pérez-Balibrea, Moreno, & García-Viguera, 2008; Sousa et al., 2007). Nevertheless, there is a wide diversity of brassica plants being within the most popular vegetables consumed all over the world. Especially regarding the properties of *Brassica* sprouts from some traditionally consumed varieties like the Portuguese Galega and Portuguese Tronchuda cabbage, the information available is scarce or inexistent.

Phenolic compounds are one of the major antioxidant compounds of *Brassica* plants (Cartea, Francisco, Soengas, & Velasco, 2010; Podsędek, 2007) and they are mainly represented by phenolic acids and flavonoids, both of which exist predominantly as conjugated structures (Soengas, Sotelo, Velasco & Cartea, 2011). The most common non-flavonoid phenolics in brassica vegetables are hydroxycinnamic acids (Lin & Harnly, 2010; Olsen, Aaby, & Borge 2009; Vallejo, Tomás-Barberán & Ferreres, 2004). Flavonols are the most widespread of the flavonoids and within the colored flavonoids, anthocyanins are the most important group (Cartea et al., 2010), commonly represented in brassica crops by pelargonidin, cyanidin, delphinidin, peonidin, petunidin and malvidin (Moreno et al., 2010; Scalzo, Genna, Branca, Chedin, & Chassaigne, 2008; Tatsuzawa, Saito, Shinoda, Shigihara & Honda, 2006). However the phenolic profile can be quite different among species and even among crops from the same species (Cartea et al., 2010). Sprouts phenolic composition depends on seed quality as well as on numerous environmental factors, including temperature, humidity and sprouting time (Yang, Basu & Ooraikul, 2001).

Organic acids also widely distributed in fruits and vegetables, originated from biochemical processes or from some microorganisms' activity, such as yeasts and bacteria (Hernández, Lobo & González, 2009). Organic acids are mainly produced in mitochondrias through the tricarboxylic acid or Krebs cycle and in a lesser extent in the glyoxysome, as

part of the glyoxylate cycle being preferentially stored in the vacuole (Lopez-Bucio, Nieto-Jacobo, Ramirez-Rodriguez & Herrera-Estrella, 2000). The most common organic acids derived from Krebs cycle are the citric, aconitic, isocitric, ketoglutaric, succinic, fumaric, malic and oxalacetic acids, with citric and malic being normally the main organic acids (Harborne, Baxter & Moss, 1999). Shikimic and quinic acids, despite not present in the Krebs cycle, are also of great interest, as precursors of aromatic compounds (Sousa et al., 2009). In plants the organic acids can act as cofactors, buffering agents, and intermediates of the most important metabolic pathways of carbohydrates, lipids, and proteins (Koyuncu, 2004). Such compounds are also extensively used as additives in food industry, namely as antioxidants (tartaric, malic, and citric), acidulants (tartaric, malic, citric, and ascorbic acids), or preservatives (sorbic and benzoic acids) (Cunha, Fernandes & Ferreira, 2002; Shui & Leong, 2002). The presence of organic acids among the constituents of some brassica crops was reported in different works (Faik Ahnmet Ayaz et al., 2006; Ferreres et al., 2006; Ferreres et al., 2007; Sousa et al., 2005; Sousa et al., 2008), being their profile also dependent on factors such as the specie, plant's age and type of plant tissue (López-Bucio, et al., 2000). Their role in photosynthesis may also determinant for the accumulation in plants, being also important factors for the organoleptic characteristics of fruit and vegetables (Vaughan & Geissler, 1997), which may influence a possible use of certain brassica sprouts as food.

Many of the plants bioactive compounds and their hydrolysis products have proven to have antimicrobial, antioxidant and anticancer properties (Gyawali & Ibrahim, 2014). There are an increasing concern about food safety owing to the rising bacterial contamination of foods, which is known to be responsible for spoilage and transmission of food-borne disease. Food spoilage due to microorganisms is one of the most important issues in food industry, aggravated by the possibility of food-borne disease issues, even more critical in ready-to-eat foods like seed sprouts since they are consumed raw. Prevention of pathogenic and spoilage microorganisms in these foods is usually achieved by chemical preservatives. In this sense, great efforts had been directed towards the identification of low-cost natural products that could replace synthetic chemicals. Numerous studies have already highlighted the potential of Brassica vegetables as a source of compounds with antibacterial activity (Faik Ahmet Ayaz et al., 2008; Begum & Poonkothai, 2013; Hu et al., 2004; Jaiswal, Abu-Ghannam & Gupta, 2012). Nevertheless, the potential antimicrobial activity of brassica sprouts is not yet fully characterized. To detect the antimicrobial activity of natural products extracts the use of the broth microdilution method is recommended to perform a fast screening of the minimal inhibitory concentration (MIC) determination (Klančnik, Piskernik, Jersek & Mozina, 2010). This was also the method used

in this work as it is more sensitive than the traditional screening agar methods, being more appropriate for a rapid quantitative determination of the antimicrobial activity of plant extracts.

The aim of this work was to characterize the main phenolic compounds and the profile of organic acids of seed sprouts from traditionally consumed brassica plants, as well as the potential antimicrobial activity of these ready to eat vegetables extracts. The correlation between the bioactive compounds and the antimicrobial activity found was also investigated.

6.2. Materials and methods

6.2.1. Reagents and Plant material

All chemicals and reagents were of analytical grade and were obtained from various commercial sources (Sigma/Aldrich and Merck). All solvents were of high-performance liquid chromatography (HPLC) grade, and all water was ultra-pure treated in a Milli-Q water purification system (Millipore, Bedford, MA, USA).

In the current study four Brassicas were selected, mostly consumed in Northern Portugal, namely Broccoli (*B. oleracea* L. var. italica Plenck, variety calabrese), Portuguese Galega (*B. oleracea* var. acephala DC), Portuguese Tronchuda cabbage (*B. oleracea* L. var. costata DC, landrace Penca da Póvoa) and red cabbage (*B. oleracea* var. capitata f. rubra). Only untreated seeds were used for sprout production. Seeds from Penca cabage and Galega kale were acquired directly from the producers in Póvoa do Varzim (North of Portugal); whereas seeds from Broccoli and red cabbage variety were supplied by Germisem - Sementes Lda.

6.2.2. Sprouting method

Sprouting was carried out according to the method described by Vale et al. (2014). The untreated seeds were previously sanitized with a sodium hypochlorite solution (0.07%, v/v) for 30 minutes, drained and washed with distilled water until they reached a neutral pH. Afterwards they were soaked in water for 12 hours in darkness, at room temperature with moderated shaking. Sowing has been done in individual trays containing an inert subtract of vermiculite (10x15x4cm) and the sprouting took place in a plant growth chamber (Fitoclima 200, Aralab, Rio de Mouro, Portugal) with controlled temperature (25ºC) and different photoperiod regimes. For green sprouts (GS) production a cycle of 16 h of light and 8 h of darkness was used. In the case of white sprouts (WS), sprouting occurred under 24 h of darkness. Sprouting process was carried out in triplicate for each, with a germination yield over 98%. As a result of the different growth caused by the photoperiods applied, GS

were harvested after 7, 9, 12 and 15 days of sprouting and WS after 5, 6, 7, 9 and 12 days. The harvested sprouts were frozen at -80ºC, freeze-dried (Scanlaf 110-4 PRO, Lynge, Denmark) powdered in a mill (Retsch ZM 200, Haan, Germany) and kept in a desiccator until analysis.

6.2.3. Analytical procedures

6.2.3.1. Phenolic compounds determination

The freeze dried samples were extracted with 70% methanol. Three replicates were extracted using a published method (Bennett, Rosa, Mellon, & Kroon, 2006). Briefly, two replicates (40 mg each) were extracted with 1 mL of 70% (v/v) methanol, and a third replicate (40 mg) was extracted with 950µL of 70% (v/v) methanol and 50µL of a solution of rutin (1mg mL^{-1}). The samples were first heated (70ºC) for 30min with vortex mixing every 5min and then centrifuged at 4ºC for 20 min at 17000g. The supernatants were collected and analyzed in a HPLC system (Thermo Surveyor HPLC), that was composed by a solvent degasser, a quaternary pump, a thermostatically controlled auto-sampler (set at 10ºC) and a column oven (set at 25ºC). The compounds were separated in a Phenomenex Luna C18 column (250 x 4.6mm i.d., 5µm) with a Phenomenex Security guard pre-column with a C18 cartridge. The mobile phase as composed by solvent A, 0.1% (v/v) TFA and solvent B, acetonitrile (0.1% (v/v) TFA), with a flow rate of 1 mL min^{-1} and an injection volume of 10 µL. The diode array detector recorded the spectra between 200 and 600 nm, and the chromatograms were also registered at 227, 270, 370 nm for flavonoids and phenolic acids monitorization, and 520 nm for anthocyanins. The compounds were tentatively identified by comparison with external standards, retention times and U.V. spectra. The phenolics in the samples were also confirmed by spiking the samples with the external standards. The results were presented as µg.g^{-1} dry weight (dw) basis.

6.2.3.2. Organic acids determination

Extraction procedure was performed according to Sousa et al. (2009) with some minor adaptations. Approximately 0.5 g of powdered freeze dried sprouts were boiled for 60 min in 25 mL of water and then filtered over a Buchner funnel. The resulting extract was lyophilized and kept in a desiccator in the dark. The extract was redissolved in 0.01 N sulphuric acid (100 mg.mL^{-1}) prior to analysis by HPLC-DAD. The chromatographic system (Jasco) was composed by a solvent degasser unit, a quaternary gradient pump and a thermostatically controlled auto-sampler. The detector was a Diode-Array with the chromatograms being registered at 214 nm and the spectra between 200 and 600nm. A volume of 20 µL was injected onto a C18 Kromasil (250x4.6 mm i.d., 5µm size) equipped

with a C18 guard column, both thermostated at 30ºC. Elution was carried out isocratically at a solvent flow rate of 0.2 mL min^{-1} using 0.005mol L^{-1} sulfuric acid solution as mobile phase. To identify and quantify the organic acids present in the samples, different external standards also injected and used to construct external calibration curves.

6.2.3.3. Bacterial strains and growth conditions

Five bacterial strains, namely *Escherichia coli* O 157: H7 ATCC 35150, *Salmonella typhimurium* ATCC 14028, *Listeria monocytogenes* ATCC 35152, *Bacillus cereus* ATCC 11778 and *Staphylococus aureus* ATCC, were used for antimicrobial activity testing. The selection of the bacterial strains aimed for the most challenging microorganisms for the safety of food products.

The cultivation/assay medium for *L. monocytogenes* and E*. coli* O157:H7 was Tryptone Soy Broth or Agar (TSB, TSA, Oxoid, Hampshire, UK); for *B. cereus* and *S. aureus* was Müeller Hinton Broth or Agar (MHB, MHA, Oxoid, Hampshire,UK).

The bacterial cultures were prepared by picking 24-h-old colony from TSA/MHA plates and suspending it in an appropriate medium (5 mL). Cultures were grown aerobically for 20 h with continuous agitation (100 rpm) at 37 °C. For antibacterial activity assays 1 mL of each culture was diluted with TSB or MHB medium to 10^5 –10^6 CFU/mL using the turbidity McFarland scale.

6.2.3.4. Antimicrobial activity by broth microdilution method

Aqueous extracts from the sprouts were obtained according to Vale et al. (2014). Briefly 0.5 g of freeze-dried sprouts were extracted twice with distilled water (final volume 50 mL), during 1h, under stirring and light protection. Then the samples were placed in an ultrasonic bath at room temperature for 20 min. Finally, the extracts were filtered (Whatman No. 1 paper), frozen at -80 ºC and lyophilized. The freeze-dried extracts were kept in desiccators, in the dark until analysis. The extracts were dissolved in milliQ purified water to a final concentration of 25 $mg.mL^{-1}$.

The broth microdilution was performed in sterile 96-well microplates according to Klančnik et al. (2010) with some minor adjustments. Sprout extracts (50 µL) were added to the first well and a serial of dilutions were made down to a minimum concentration, ranging between 25 -2.5$mg.mL^{-1}$. Each bacterial suspension (50 µL) in suitable growth medium was then added to the 96-well microplate. For positive controls, a bacterial suspension in an appropriate growth medium and a bacterial suspension in an appropriate growth medium with ethanol, both in the highest quantity present in the broth microdilution assay was added to the 96-well microplate. Negative controls correspond to the growth medium and plant extract or growth medium and sinapic acid as a pure phenolic acid.

The content of each well was mixed on a microplate shaker (Titramax 1000) at 900 rpm for 1 min followed by incubation for 24h at 37 ^{o}C. The MIC corresponded to the lowest concentration where no viability was observed after 24h on the basis of metabolic activity (Mourey & Canillac, 2002). The presence of respiratory activity was detected by the development of red color in the wells after adding 10 µL/well of a 2 mg.mL^{-1} solution of INT (2-p-iodophenyl-3-p-nitrophenyl-5-phenyl tetrazolium chloride), and incubated at room temperature for 30 min in the dark (Eloff, 1998). The MIC^{INT} was determined as the lowest sample concentration at which no red color (significant live growth) appeared. All measurements of MIC values were done in triplicate.

6.2.4. Statistical analysis

Data obtained from the study were presented as mean ± standard deviation and the differences between samples and growth conditions were tested by one-way ANOVA followed by post-hoc Tukey comparison tests. Statistical significance was defined for $p < 0.05$. Correlation coefficients (r) to determine the relationship between variables were calculated using the Bivariate correlation statistical function. All analyses were made using the SPSS 15.0 software (SPSS Inc., Chicago, Illinois, EUA) for Windows.

6.3. Results and discussion

6.3.1. Phenolic compounds

The phenolic compounds found in the brassica sprouts extracts were divided in two different classes, the hydroxycinnamic acids derivatives and anthocyanins, accordingly to their UV-Vis spectra and the information available in published literature (Ferreres et al., 2007; Sousa et al., 2007). The majority of the compounds showed a maximum absorbance at 330 nm, characteristic of hydroxycinnamic acids (Ferreres et al., 2007), being these compounds described as the main component of the phenolic profile of other sprouts and seeds from *B. oleracea* varieties (Ferreres et al., 2007; Pająk, Socha, Gałkowska, Rożnowski, & Fortuna, 2014; Sousa et al., 2007). Although flavonoids are described as the most common phenolic compounds of several brassica vegetables (Cartea et al., 2010), the seeds and sprouts of those vegetables have usually a higher content of phenolic acids (Ferreres et al., 2007; Pająk et al., 2014; Sousa et al., 2007). Through the comparison of their UV-vis spectra with the available external standards, the brassica sprouts revealed a prevalence of sinapic acid derivatives that accordingly to the published works corresponded mainly to sinapoyl glucosides (Ferreres et al., 2007; Sousa et al., 2007). For this, the phenolic acids were quantified using the sinapic acid calibration curve, and the results presented as µg of hydroxycinnamic acids per g (dw) of sprout (see Figure 6.1). Sprouts

from Penca cabbage showed the highest mean content in hydroxycinnamic acids (251.9 µg.g^{-1} (dw)) followed by red cabbage (225.5 µg.g^{-1} (dw)), Galega kale (222.3 µg.g^{-1} (dw)) and broccoli (213.2 µg.g^{-1} (dw)). The values encountered are within the same range of those presented for the phenolic acids content of other vegetable sprouts (Pająk et al., 2014).

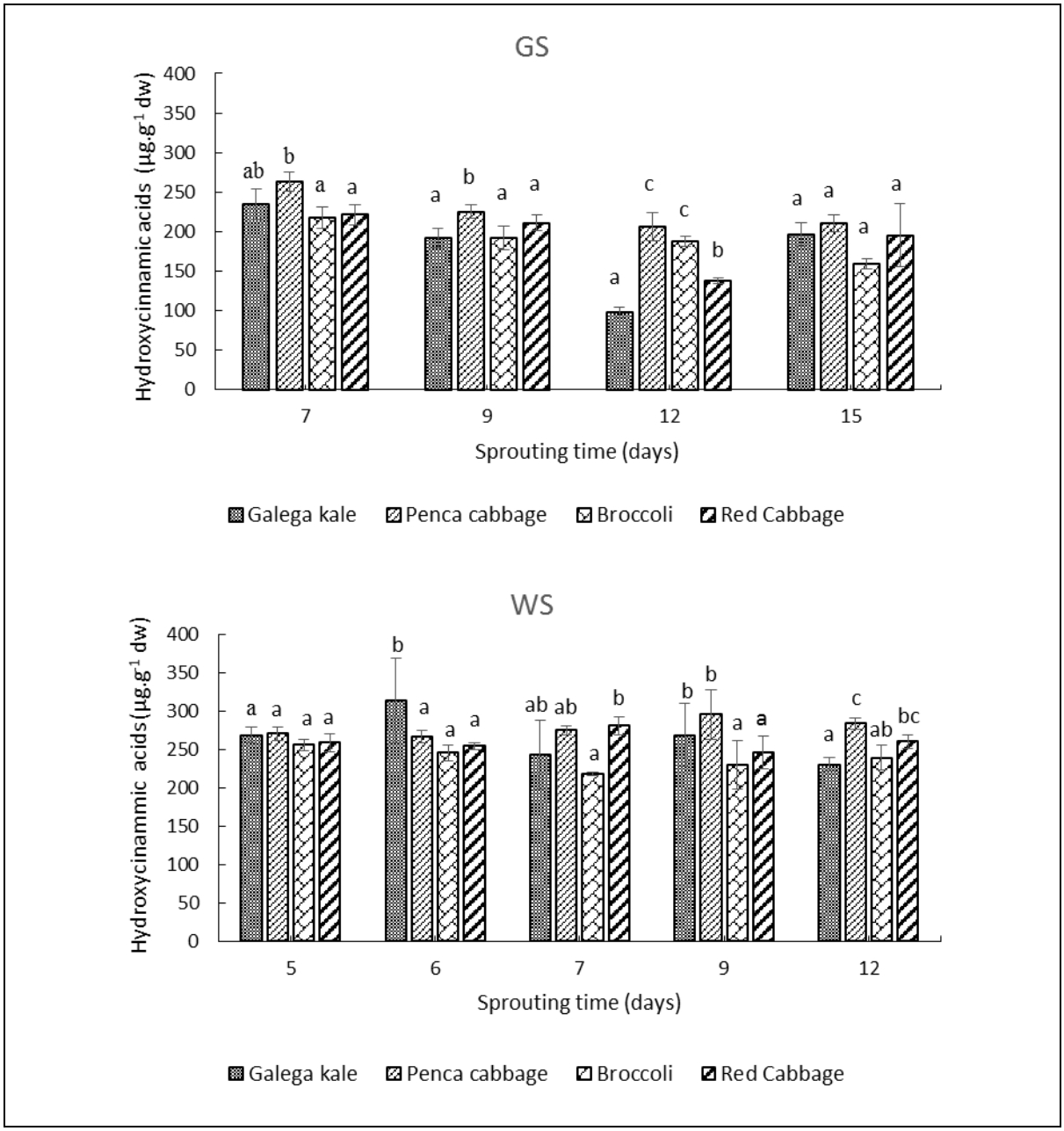

Figure 6.1 Variations in hydroxycinnamic acids concentration (µg.g-1 dw) during sprouting time, of sprouts from four brassica varieties, grown under light (GS) and darkness (WS) condition. The data represent the mean of three replicates. Different letters within the same *B. oleracea* variety represent a significant difference ($p < 0.05$) between samples from different sprouting periods.

The type of photoperiod used for sprout production had a significant effect in hydroxycinnamic acids content, with sprouts produced under light/darkness cycles showing

in general a lower level (less 32%) than sprouts produced under dark. Galega kale sprouts were the most influenced by light exposure, showing the highest difference between GS and WS at the 12th day of sprouting, with WS having about the double of the phenolic acids content found in the GS sprouts. On the other hand, the phenolic acids content of Broccoli sprouts was the less affected by the different spouting conditions, showing smaller differences between GS and WS at the same sprouting time. The results obtained for the hydroxycinnamic acids content of broccoli sprouts where however contrary to the results reported by Pérez-Balibrea et al. (2008), whose sprouts showed a higher phenolic content when sprouted under light/darkness cycles.

Sprouting time also influenced significantly ($p<0.05$) the concentration of hydroxycinnamic acids, especially between the days seven and twelve. The preservation of hydroxycinnamic acids during sprouting was higher in WS than in GS, having been observed an average decrease of 33%, between the 7th and the 12th day in light produced sprouts and 6% in sprouts growth without light. The main losses between the 7th and the 15th day of sprouting occurred in Galega kale GS (59%), followed by Red cabbage GS (38%) and Penca cabbage GS (22%). The decreasing of the hydroxycinnamic acids over the time could be a result of their role in cell wall biosynthesis and antioxidant reactions (Sousa et al., 2007).

Concerning the identification of anthocyanins, this class of compounds was only detected in red cabbage sprouts. Once more, the effect of different photoperiods used during sprouting was determinant with the red cabbage GS having an average level of 15.2 $\mu g.g^{-1}$ (dw) and the WS of 7.6 $\mu g.g^{-1}$ (dw). The anthocyanins correspond to the major red, blue and purple pigments of plants and are known for their capacity to absorb visible and UV radiation and for providing effective antioxidant protection. One of their possible role in plants metabolism is the protection of the is photosynthetic apparatus from the effects of excessive incident visible or UV-B radiation and photooxidative stress, which may lead the plant to synthetize more anthocyanins in the presence of light (Quina et al., 2009). In the red cabbage sprouts where identified four different glycosylated anthocyanins, the cyanidin-3-glucoside, the peonidin-3-glucoside, the malvidin-3-glucoside and the malvidin-3-galactoside (see Figure 6.2). Malvidin-3-glucoside was the main anthocyanin present in red cabbage sprouts, accounting for 60% and 73 % of the total anthocyanins in GS and WS, respectively. Peonidin-3-glucoside was the second, showing an average level of 17 $\mu g.g^{-1}$ (dw) in GS and 4.0 $\mu g.g^{-1}$ (dw) in WS, followed by malvidin-3-galactoside (5 $\mu g.g^{-1}$ (dw) and 3 $\mu g.g^{-1}$ (dw) in GS and WS, respectively) and finally by cyanidin-3-glucoside (2 $\mu g.g^{-1}$ (dw) and 1 $\mu g.g^{-1}$ (dw) in GS and WS, respectively). Contrary to the marked evolution pattern

seen in the hydroxycinnamic acids content over the sprouting period, the anthocyanins level revealed a lower variation between the different sprouting times.

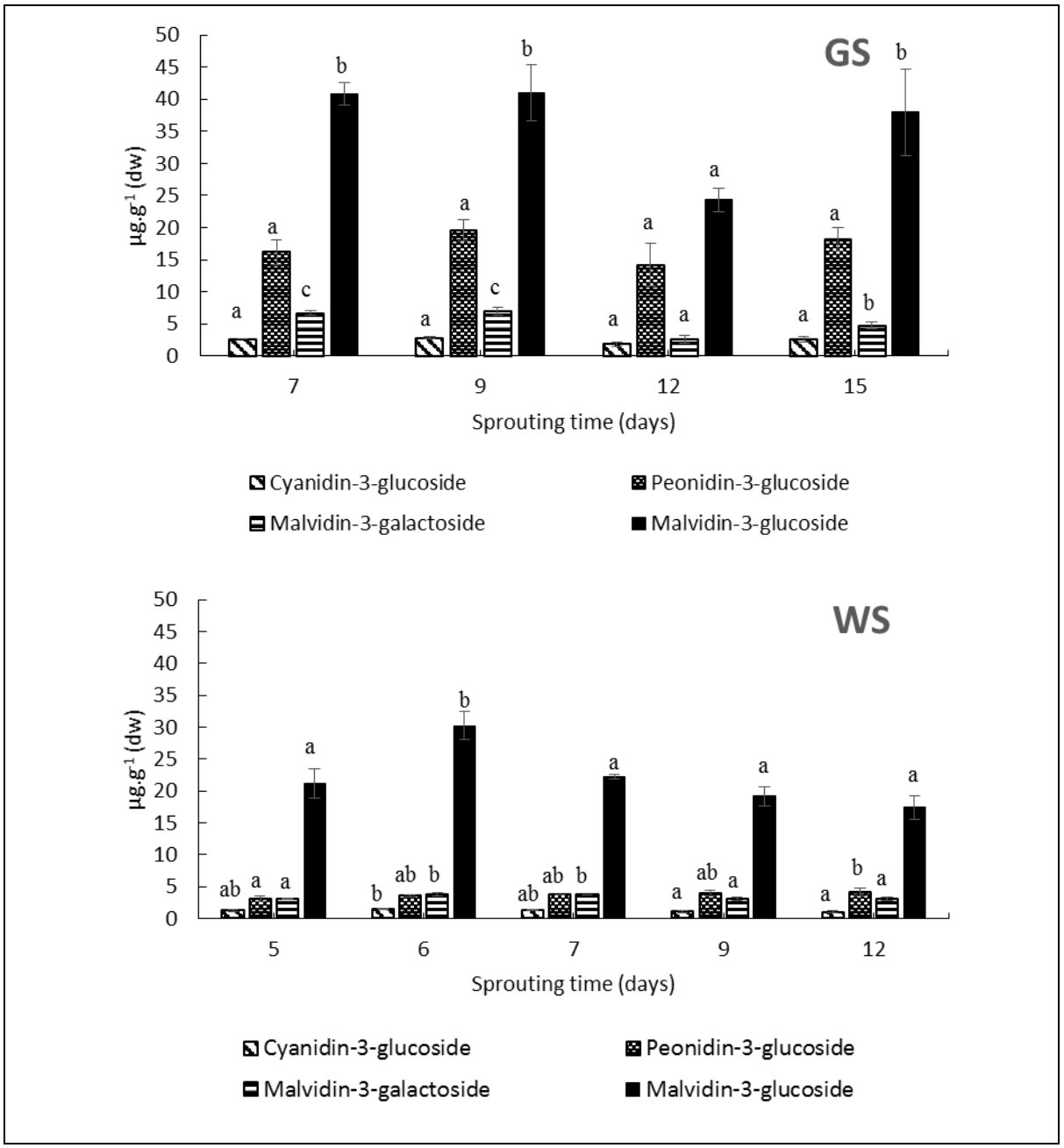

Figure 6.2 Anthocyanins content in red cabbage sprouts, grown under light (GS) and darkness (WS) conditions. The data represent the mean of three replicates. Different letters within the same compound column represent a significant difference ($p < 0.05$) between different sprouting periods.

6.3.2. Organic acids profile

The screening of organic acids showed the presence of oxalic, malic, maleic, aconitic, ascorbic, shikimic, citric and fumaric acids in the different sprouts extracts (Table 6.1). Citric, oxalic and malic acids were the major organic acids found in sprouts of all the

varieties accounting for 67%, 19% and 13% of the total organic acids, respectively. Citric and malic acids are known for being present in large amounts in all plant materials (seeds and leaves) (Fernandes, 2011) as they accumulated in plants tissues (Harborne et al., 1999). In the other hand, the presence of oxalic acid was not verified in the work presented by Sousa et al. (2007) that described an otherwise similar composition of the other organic acids for their Penca cabbage sprouts. The synthesis and intracellular accumulation of oxalic acid in plants is related to the cellular calcium homeostasis (Kidd, Llugany, Poschenrieder, Gunse & Barcelo, 2001). However, from a nutritional perspective, oxalic acid is considered an antinutrient, since if ingested in large quantities can reduce the bioavailability of calcium and sometimes other minerals (Franceschi & Nakata, 2005).

Table 6.1 Quantification of organic acids in Brassica sprouts (mg.g-1 dw, dry basis) grown under light (GS) and dark condition (WS) and at different sprouting times. Results are expressed as mean ± standard deviation of three determinations. Values sharing the same letter within the column and photoperiod are not significantly different ($p \leq 0.05$). n.d., not detected.

		Organic acids (mg.g^{-1} dw)							
	Sprouting Time	Aconitic acid	Ascorbic acid	Shikimic acid	Citric acid	Fumaric acid	Maleic acid	Malic acid	Oxalic acid
		GS							
Galega kale	7	1.0±0.02^{a}	0.57±0.01^{a}	nd	17.13±0.27^{a}	0.05±0.002^{a}	2.35±0.004^{a}	4.89±0.36^{a}	22.72±0.32^{a}
	9	2.05±0.01^{c}	0.72±0.02^{b}	nd	21.5±0.19^{b}	0.06±0.001^{b}	4.33±0.01^{b}	15.23±0.15^{b}	42.95±0.39^{b}
	12	1.98±0.01^{b}	3.78±0.02^{c}	nd	21.74±0.24^{b}	0.08±0.001^{c}	5.16±0.02^{c}	30.09±0.29^{c}	45.08±0.07^{c}
	15	3.13±0.01^{d}	4.33±0.02^{d}	nd	24.49±0.14^{c}	0.10±0.004^{d}	6.22±0.08^{d}	61.06±0.86^{d}	88.00±0.81^{d}
Penca cabbage	7	2.00±0.01^{a}	0.39±0.004^{a}	0.9±0.004^{d}	36.32±0.02^{a}	0.04±0.001^{a}	5.09±0.024^{a}	1.46±0.09^{a}	14.22±0.24^{a}
	9	2.6±0.01^{c}	2.03±0.03^{b}	0.66±0.03^{c}	48.57±0.2^{c}	0.05±0.001^{b}	5.67±0.01^{b}	5.04±0.02^{b}	40.24±0.26^{b}
	12	2.38±0.02^{b}	1.98±0.02^{ab}	0.47±0.02^{b}	43.84±0.01^{b}	0.06±0.001^{c}	6.13±0.02^{c}	6.49±0.1^{c}	79.78±1.44^{c}
	15	2.79±0.02^{d}	0.96±0.02^{ab}	0.1±0.01^{a}	85.71±0.29^{d}	0.07±0.003^{d}	7.35±0.01^{d}	7.34±0.03^{c}	96.22±0.39^{d}
Broccoli	7	0.84±0.004^{a}	0.83±0.003^{a}	2.00±0.02^{d}	680.83±1.18^{a}	0.04±0.001^{a}	4.25±0.02^{c}	2.54±0.01^{a}	32.1±0.23^{a}
	9	1.2±0.003^{b}	0.91±0.01^{b}	1.70±0.02^{c}	775.49±0.9^{b}	0.05±0.001^{b}	3.72±0.03^{b}	25.15±1.46^{b}	75.91±0.26^{b}
	12	1.9±0.01^{c}	0.95±0.01^{c}	0.55±0.06^{b}	783.21±0.11^{c}	0.07±0.002^{c}	5.31±0.34^{d}	30.22±0.41^{c}	118.88±1.35^{c}
	15	2.2±0.03^{d}	3.13±0.02^{d}	0.05±0.001^{a}	831.06±0.26^{d}	0.09±0.006^{d}	0.69±0.005^{a}	69.28±0.60^{d}	139.62±1.54^{d}
Red cabbage	7	2.04±0.005^{a}	0.31±0.1^{a}	nd	422.15±1.00^{a}	0.04±0.001^{a}	3.24±0.005^{a}	9.00±0.06^{a}	17.15±0.28^{a}
	9	2.17±0.02^{b}	0.30±0.12^{a}	nd	593.45±1.40^{b}	0.05±0.005^{b}	3.4±0.02^{a}	11.53±0.39^{b}	29.61±0.23^{b}
	12	3.92±0.02^{d}	0.32±0.01^{a}	nd	623.26±1.72^{d}	0.07±0.003^{c}	4.82±0.27^{d}	16.23±0.29^{c}	43.42±0.39^{c}
	15	3.6±0.003^{c}	0.33±0.01^{a}	nd	604.88±0.77^{c}	0.08±0.003^{d}	4.25±0.29^{c}	18.37±0.25^{d}	48.96±0.1^{d}
		WS							
Galega kale	5	3.026±0.1^{b}	0.63±0.01^{a}	1.18±0.02^{b}	nd	nd	5.65±0.03^{a}	3.51±0.005^{a}	123.41±0.79^{a}
	6	2.90±0.1^{a}	0.79±0.01^{b}	1.15±0.01^{b}	nd	nd	6.07±0.04^{b}	5.48±0.12^{b}	139.32±5.51^{a}
	7	4.31±0.1^{c}	1.37±0.02^{c}	0.40±0.01^{a}	nd	nd	6.80±0.10^{c}	8.08±0.21^{c}	182.32±0.57^{b}
	9	4.80±0.01^{d}	2.25±0.05^{d}	0.26±0.02^{a}	nd	nd	8.59±0.02^{d}	11.53±0.27^{d}	271.27±2.79^{c}
	12	5.87±0.02^{e}	2.35±0.02^{e}	0.34±0.43^{a}	nd	nd	9.72±0.12^{e}	20.63±0.30^{e}	335.62±2.86^{d}
Penca cabbage	5	0.75±0.01^{a}	0.55±0.04^{a}	0.77±0.01^{e}	nd	nd	1.08±0.004^{ab}	120.61±2.28^{a}	63.98±1.11^{a}
	6	0.93±0.01^{b}	0.89±0.01^{b}	0.62±0.02^{d}	nd	nd	1.44±0.03^{ab}	163.16±0.97^{b}	83.02±0.71^{c}
	7	1.20±0.08^{c}	1.01±0.01^{c}	0.53±0.001^{c}	nd	nd	1.46±0.01^{ab}	177.70±0.51^{c}	83.36±0.58^{c}
	9	1.32±0.01^{d}	1.32±0.01^{d}	0.37±0.01^{b}	nd	nd	0.63±0.01^{a}	223.82±1.45^{d}	80.94±0.32^{b}
	12	1.48±0.01^{e}	1.92±0.01^{e}	0.23±0.003^{a}	nd	nd	2.03±0.01^{b}	238.96±2.09^{e}	150.85±0.49^{d}
Broccoli	5	0.58±0.01^{a}	0.80±0.003^{a}	2.30±0.02^{e}	438.27±0.43^{a}	nd	0.80±0.01^{a}	5.18±0.11^{a}	31.52±0.41^{b}
	6	0.73±0.034^{a}	0.80±0.01^{a}	2.05±0.01^{d}	521.90±0.16^{b}	nd	0.99±0.01^{b}	107.52±1.07^{b}	30.85±0.09^{a}
	7	0.72±0.01^{a}	1.09±0.01^{b}	1.51±0.05^{c}	605.75±1.09^{c}	nd	1.72±0.01^{c}	117.30±1.49^{c}	44.93±0.20^{c}
	9	1.25±0.01^{b}	3.19±0.03^{d}	0.32±0.01^{b}	709.54±0.43^{d}	nd	2.33±0.01^{d}	178.51±0.36^{d}	53.63±0.23^{d}
	12	1.18±0.01^{b}	3.10±0.01^{c}	0.03±0.01^{a}	590.59±3.36^{e}	nd	3.29±0.01^{e}	176.22±1.66^{d}	105.09±0.65^{e}
Red cabbage	5	1.66±0.01^{b}	0.51±0.01^{a}	1.39±0.01^{e}	345.59±8.48^{a}	0.03±0.00^{a}	2.80±0.08^{c}	13.36±0.03^{a}	23.73±0.32^{a}
	6	1.48±0.002^{a}	1.14±0.002^{b}	0.81±0.03^{d}	361.97±0.19^{b}	0.05±0.00^{b}	2.56±0.05^{b}	14.68±0.08^{b}	33.24±0.42^{b}
	7	1.83±0.01^{c}	1.50±0.004^{b}	0.46±0.01^{c}	443.82±0.42^{c}	0.22±0.02^{d}	2.29±0.05^{a}	108.70±0.29^{d}	59.23±0.51^{d}
	9	2.45±0.003^{d}	2.11±0.01^{c}	0.36±0.01^{b}	524.07±0.38^{e}	0.06±0.00^{bc}	2.86±0.03^{c}	102.34±0.37^{c}	57.63±0.28^{c}
	12	2.39±0.002^{e}	2.35±0.05^{c}	0.17±0.002^{a}	512.56±0.68^{d}	0.07±0.001^{c}	4.43±0.03^{d}	116.17±0.77^{e}	202.83±0.93^{e}

Light exposure and sprouting time had a significant effect on organic acid composition of the sprouts ($p<0.05$), being that influence dependent on the brassica variety and organic acid analyzed. In the case of citric acid, the absence of light during growth dictated the absence of a detectable amount of this acid in the two Portuguese brassica varieties, and also an inferior mean content in the other two varieties studied (losses between 17% and 20%). Regarding the accumulation of oxalic acid the opposite behavior was seen for Galega kale, Penca cabbage and Red cabbage sprouts, with the WS showing a greater content than the GS during the same growth period (more 78%, 42% and 61%, respectively). The same was seen in malic acid, that showed a higher accumulation in WS of Penca cabbage, Broccoli and Red cabbage (more 97%, 78% and 84% mean content, respectively) than the correspondent GS. In the other minor constituents of the organic acid profile of the sprouts the use of the light/darkness cycles did not show the same pronounced effect as it was registered for the major organic acids. However, some exceptions were seen in fumaric acid, whose presence was not detected in the WS of Galega kale, Penca cabbage and Broccoli and also in shikimic acid, that was not present in Galega Kale and Red cabbage GS.

The total organic acids content increased significantly over the sprouting time, ranging from 393.3 to 546.6 $mg.g^{-1}$ (dw) between the same sprouting days. In total, the content of organic acids increased 52% in the WS and 39% in the GS. Individually, each organic acid analyzed showed great increase during the studied sprouting period, representing for the oxalic, malic and ascorbic acids an increment of more than 100% of the initial content in both GS and WS. This behavior could be expected as a result of an increased metabolic activity during germination, which rapidly resumes the glycolytic and the tricarboxylic acid cycle and the β-oxidation of fatty acids after germination (Pracharoenwattana, Cornah & Smith, 2005; Li, Wu, Tsang & Cutler, 2005). Although the effect of the germination time was clear in the obtained results, the same was not described in other work about the organic acids content of Penca cabbage sprouts (Sousa et al., 2007), where the levels of citric, oxalic, aconitic and fumaric acids remained constant, considering the same germination period. The only exception in the general increasing behavior was found in the evolution of the shikimic acid, that showed lower content in sprouts with longer sprouting times. Also, in some of the quantified organic acids in Broccoli and red cabbage sprouts, the levels found after 15 (in GS) and 12 (in WS) days was significant ($p<0.05$) inferior to the ones found in the samples from the previous germination time (please see citric, maleic and ascorbic acid levels at the last sampling day in Table 6.1), probably as a result of the long sprouting period and the onset of some senescence reactions on the sprouts.

The variety of the brassica sprout was also determinant to the profile of organic acids encountered, showing, in the same conditions, very distinct levels between the four varieties tested. Galega kale WS produced in dark conditions showed the highest levels of oxalic acid among all sprouts studied, being this acid the principal compound of the organic profile of both GS and WS of Galega kale. In addition to oxalic acid, the main composition of the sprouts from this variety included also malic acid (11.5±6.6 $mg.g^{-1}$ (dw)), maleic acid (7.8±1.7 $mg.g^{-1}$ (dw)) and aconitic acid (4.5±1.2 $mg.g^{-1}$ (dw)). Citric and fumaric acid were not detected in Galega kale and in Penca cabbage WS, showing in the sprouts produced under light a citric acid content very inferior (more than 10 times) to the ones found in Broccoli and Red cabbage sprouts (see Table 6.1). In the Penca cabbage sprouts, malic acid was the main organic acid in the sprouts grown under darkness, being the oxalic acid the main component of the Penca Cabbage sprouts produced under light/darkness cycles (see Table 6.1). Sousa et al. (2007) described a somewhat different composition for the organic acids profile of Penca cabbage sprouts, showing a lower levels of oxalic acid, which only represented a maximum of 4% of the total organic acids quantified, and showing also a predominance of citric and malic acids in the profile of their sprouts. Broccoli sprouts showed to be a good source of citric acid (mean content of 607±100 $mg.g^{-1}$ (dw)) accounting for 74.3% of the total, followed by malic and oxalic acid but in lower levels. Regarding red cabbage WS the main organic acids quantified were citric acid (461±83 $mg.g^{-1}$ (dw), accounting for 72% of the total, followed by oxalic acid and malic acid, representing 14% and 13% of the total, respectively.

6.3.3. Antimicrobial effect of brassica sprout extracts

All analyzed extracts showed a notable antimicrobial activity against the tested microorganisms (see Table 6.2), showing a significant inhibition of the growth of those microorganisms that represent a major concern for the safety of food products. Among the brassica varieties studied, broccoli and red cabbage extracts showed the highest antibacterial activity against most of the tested microorganisms showing the lowest MIC^{INT} values (see Table 6.2). The high antibacterial activity of broccoli plants was also reported by Jaiswal et al. (2012), when was compared to other *B. oleracea* varieties.

The resistance to the extracts was not correlated with the microorganisms specie, as *E. coli* (a Gram negative strain) was the most sensitive bacteria (7 $mg.mL^{-1}$ MIC^{INT}) followed by *L. monocytogenes*, *S. aureus* and *B. cereus* (a Gram positive, with an average MIC^{INT} of 12 $mg.mL^{-1}$). The most resistant bacteria to the sprouts extracts was *S. typhimurium*, a Gram negative microorganism, which showed an average MIC^{INT} of 13 $mg.mL^{-1}$. *E.coli* and *L. monocytogenes* were more sensitive to broccoli and red cabbage extracts (5 and 7 $mg.mL^{-1}$ MIC^{INT}, respectively). Contrary, Galega kale was the less effective

extract against *E. coli* (14 mg.mL^{-1} MIC^{INT}) and Penca cabbage against *L. monocytogenes* (14 mg.mL^{-1} MIC^{INT}). Red cabbage and Broccoli extracts were also the most effective against *S. aureus* (6 mg.mL^{-1} MIC^{INT}), being the best antimicrobial activity against *B. cereus* exhibited by red cabbage (8 mg.mL^{-1} MIC^{INT}) and Penca cabbage (10 mg.mL^{-1} MIC^{INT}) extracts.

Table 6.2 Antimicrobial activity of *Brassica* sprouts extracts, expressed as MIC INT (mg/mL), determined by broth microdilution method for gram-negative and gram-positive bacteria.

Sprouting time (days)		**MIC ^{INT}** (mg.mL^{-1})				
		E. coli	L. monocytogenes	S. typhimurium	B. cereus	S. aureus
	GS					
Galega kale	**7**	7	10	9	20	20
	9	20	20	20	20	20
	12	20	10	20	20	25
	15	8	20	20	20	20
		b	b	b	c	c
Penca cabbage	**7**	5	7	9	10	6
	9	5	10	20	10	7
	12	7	20	10	9	20
	15	8	20	20	9	25
		a	b	b	b	b
Broccoli	**7**	2.5	5	5	9	5
	9	5	6	7	9	6
	12	5	5	6	7	6
	15	6	6	7	7	7
		a	a	a	a	a
Red cabbage	**7**	6	5	6	8	5
	9	5	5	6	8	5
	12	5	5	6	8	6
	15	6	5	6	6	6
		a	a	a	a	a
	WS					
Galega kale	**5**	20	20	25	10	8
	6	20	20	25	10	25
	7	20	25	25	20	25
	9	5	9	20	20	20
	12	6	20	20	20	20
		b	b	d	b	c
Penca cabbage	**5**	9	20	20	9	5
	6	9	20	20	10	5
	7	20	25	9	9	25
	9	9	20	20	10	25
	12	6	20	20	9	8
		ab	b	c	a	b
Broccoli	**5**	5	5	7	20	6
	6	6	9	6	20	8
	7	6	6	8	20	6
	9	5	6	7	9	8
	12	5	5	5	8	5
		a	a	a	b	a
Red cabbage	**5**	5	5	6	8	5
	6	5	2.5	5	8	5
	7	6	8	20	8	8
	9	5	9	20	6	8
	12	20	20	8	9	7
		a	a	b	a	a

Within each column, the different letters mean significantly differences between the brassica varieties produced under the same photoperiod type, at $p<0.05$.

The sprouting conditions used were determinant for the encountered antimicrobial activity against *L. monocytogenes* and *S. typhimurium* ($p<0.05$), with sprouts produced under light conditions showing a global higher antimicrobial activity. For the other microorganisms there were no significant ($p>0.05$) differences between sprouts produced under light or dark conditions. The sprouting time did not have also a significant effect ($p>0.05$) in the antimicrobial activity of the sprout extracts, being the main differences observed assigned to the type of brassica variety.

A correlation between the antimicrobial activity and organic acids and phenolic composition of brassica sprouts was also studied, being the significant Pearson's correlation coefficients presented in Table 6.3. Although only a few strong correlations were achieved ($0.8 \leq r < 1$), several significant moderate correlations ($0.5 \leq r < 0.8$), at 0.01 confidence level were found, namely between the anthocyanins present in red cabbage extracts and the MIC^{INT} found for *E. coli*, *L. monocytogenes*, *S. typhimurium* and *Staphylococcus aureus*. The highest correlation coefficient (0.681) was achieved between *Salmonella* MIC^{INT} and Peonidin-3-glucoside content of the red cabbage extracts. The hydroxycinnamic acids content of Penca cabbage extracts was also moderately correlated (0.658) with *L. monocytogenes* MIC^{INT}. The antibacterial activities of the sprouts extracts showed also several significant correlations ($p<0.01$ and $p<0.05$) with the different organic acids, showing a wide range of weak and moderate correlations and even some strong correlation coefficients (see Table 6.3). Galega kale extracts showed a strong correlation coefficient (0.912) between shikimic acid content and *B. cereus* MIC^{INT}, while Penca cabbage extracts reveal a higher correlation coefficient (0.709) between oxalic acid and *L. monocytogenes* MIC^{INT}. Strong correlations between organic acids and the antibacterial activity of the extracts were also found in broccoli and red cabbage sprouts. Broccoli extracts were rich in citric acid which was highly correlated with the potential of broccoli to inhibit the growth of *B. cereus*. Red cabbage extracts showed one of the highest antimicrobial activities, which were also strongly correlated with the different organic acids encountered in these sprout variety. Oxalic acid was highly correlated with the potential of the red cabbage extracts to inhibit *E. coli* and *L. monocytogenes* growth, while malic acid showed a strong correlation with the inhibition of the *S. aureus* growth.

Table 6.3 Significant correlations between the organic acids and phenolic compounds with the antimicrobial activity of *Brassica* sprouts extracts.

Phenolic Compounds		Pearson's correlation coefficients
Penca cabbage	*L. monocytogenes* * Hydroxycinnamic acids	- 0.658**
	B. cereus * Hydroxycinnamic acids	0.415*
Broccoli	*L. monocytogenes* * Hydroxycinnamic acids	0.405*
	B. cereus * Hydroxycinnamic acids	0.470*
Red cabbage	*E. coli* * Cianidin-3-glucoside	0.436*
	E. coli * Malvidin-3-glucoside	0.424*
	L. monocytogenes * Cyanidin-3-glucoside	0.499**
	L. monocytogenes * Peonidin-3-glucoside	0.394*
	L. monocytogenes * Malvidin-3-galactoside	0.428*
	L. monocytogenes * Malvidin-3-glucoside	0.491**
	S. typhimurium * Cyanidin -3-glucoside	0.509**
	S. typhimurium * Peonidin-3-glucoside	0.686**
	S. typhimurium * Malvidin-3-galactoside	0.552**
	S. typhimurium * Malvidin-3-glucoside	0.523**
	S. aureus * Cyanidin-3-glucoside	0.576**
	S. aureus * Peonidin-3-glucoside	0.681**
	S. aureus * Malvidin-3-galactoside	0.585**
	S. aureus * Malvidin-3-glucoside	0.585**
Correlation with Organic Acids		
Galega kale	*E. coli* * Shikimic acid	0.402*
	E. coli * Oxalic acid	-0.424*
	L. monocytogenes * Shikimic acid	0.381*
	S. typhimurium * Aconitic acid	0.454*
	S. typhimurium * Shikimic acid	0.626**
	S. typhimurium * Citric acid	-0.506**
	S. typhimurium * Fumaric acid	-0.409*
	S. typhimurium * Maleic acid	0.492**
	B cereus * Ascorbic acid	0.461*
	B cereus * Shikimic acid	-0.912**
	B cereus * Citric acid	0.472*
	B cereus * Fumaric acid	0.451*
	B cereus * Malic acid	0.412*

Phenolic Compounds		Pearson's correlation coefficients
Penca cabbage	*E. coli* * Aconitic acid	-0.442*
	E. coli * Citric acid	-0.387*
	E. coli * Fumaric acid	-411*
	E. coli * Maleic acid	-0.448*
	E.coli * Malic acid	0.411*
	L. monocytogenes * Aconitic acid	-0.444*
	L. monocytogenes * Shikimic acid	-0.557**
	L. monocytogenes * Maleic acid	-0.456*
	L. monocytogenes * Malic acid	0.593**
	L. monocytogenes * oxalic acid	0.709**
	S. typhimurium * Shikimic acid	0.404*-
	S. typhimurium * Oxalic acid	0.404*
	B cereus * Shikimic acid	0.443*
	B cereus * Oxalic acid	-0.562**
	S. aureus * Shikimic acid	-0.620**
Broccoli	*E. coli* * Maleic acid	-0.600**
	E.coli * Malic acid	0.418*
	L. monocytogenes * Maleic acid	-0.480*
	S. typhimurium * Maleic acid	-0.463*
	B cereus * Acotinic acid	-0.744**
	B cereus * Ascorbic acid	-0.516**
	B cereus * Shikimic acid	0.695**
	B cereus * Citric acid	-0.891**
	B cereus * Fumaric acid	-0.647**
	B cereus * Maleic acid	-0.610**
	B cereus * Oxalic acid	-0.710**
	S. aureus * Maleic acid	-0.533**
Red cabbage	*E. coli* * Ascorbic acid	0.600**
	E. coli * Maleic acid	0.422*
	E.coli * Malic acid	0.565**
	E. coli * Oxalic acid	0.965**
	L. monocytogenes * Ascorbic acid	0.755**
	L. monocytogenes * Malic acid	0.785**
	L. monocytogenes * Oxalic acid	0.963**
	S. typhimurium * Ascorbic acid	0.619**
	S. typhimurium * Fumaric acid	0.382*
	S. typhimurium * Maleic acid	-0.460*
	S. typhimurium * Malic acid	0.785**
	S. aureus * Ascorbic acid	0.744**
	S. aureus * Fumaric acid	0.387*
	S. aureus * Malic acid	0.915**
	S. aureus * Oxalic acid	0.480*

*Correlation is significant at the 0.01 level. **Correlation is significant at the 0.05 level.

6.4. Conclusions

The results obtained in the present investigation indicate that brassica sprouts can be considered as good dietary source of natural phenolic compounds and organic acids. The sprouts also showed a high or moderate antimicrobial activity against some of the most challenging microorganism for guaranteeing the safety of food products. This hints at the possibility that sprout extracts can be used as alternative food preservatives and might be applicable in food industry to enhance the safety and quality of foods. The study demonstrated also that sprouts from different brassica varieties have different degrees of antimicrobial activity being red cabbage and broccoli sprouts the more effective against the studied microorganisms. However, the information available on these topics is very scarce and more research is needed to characterize the effect of sprouts from these and other brassica varieties on different microorganisms.

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CAPÍTULO 7

Effect of refrigerated storage on the bioactive compounds and microbial quality of *Brassica oleracea* sprouts

Postharvest Biology and Technology
Submited

Effect of refrigerated storage on the bioactive compounds and microbial quality of *Brassica oleracea* sprouts

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Abstract

Brassica sprouts are recognized as a healthy and convenient food product that could help to improve the numbers of vegetable consumption worldwide. However, due to great number of species and varieties, the knowledge about the bioactive composition and quality stability during storage of different *B. oleraceae* sprouts is still scarce. In this work, the glucosinolate and phenolic profile of four of four varieties of *Brassica oleracea* sprouts (red cabbage, broccoli, Galega kale and Penca cabbage) grown under light/darkness cycles and complete darkness was monitored during 12 days of storage (at 4˚C). Their microbial quality was assessed by monitoring the microbial load (mesophilic bacteria, total coliforms, yeasts and molds and presence of Salmonella) and the biogenic amines content, to guaranty the safety of this product. As expected, the content of the bioactive compounds monitored suffered significant ($p<0.05$) changes during storage, being those changes more intense after 7 days of storage. The two Portuguese varieties (Galega kale and Penca cabbage) showed to be a good source of aliphatic glucosinolates (as glucoraphanin and sinigrin), having the sprouts grown under darkness a more stable content of these compounds. The phenolic profile was characterized by a prevalence of hydroxycinnamic acids derivatives, being the content higher in the all sprouts grown under darkness. Regarding the microbial quality, the sprouts did not showed any pathogenic contamination and were considered as safe product during the studied period.

Keywords: brassica sprouts; glucosinolates, phenolic compounds, microbial quality, storage.

7.1. Introduction

A diet rich in cruciferous vegetables has been recognized to reduce the risk of different cancers, based on the effect of the breakdown products of glucosinolates (GLs). These are characteristic compounds found in all the economically important Brassicaceae crops. There are three major categories of GLs, the aliphatic, indolic and aromatic glucosinolates (Sonderby et al., 2010; Yan and Chen, 2007) being the aliphatic-GLs the most important group in cancer prevention (Fahey et al., 1997). In addition, Brassicaceae crops are also rich in other phytochemicals, like phenolic compounds that also play an important role in human nutrition. Phenolic compounds can be classified in flavonoids (flavonols, flavones, flavan-3-ols, anthocyanidins, flavanones, isoflavones and others) and non-flavonoids (phenolic acids, hydroxycinnamates, stilbenes and others) (Crozier et al., 2007), both of which exist predominantly as conjugated structures (Lee et al., 2011). The most widespread and diverse group of polyphenols in *Brassica* species are the flavonoids (mainly flavonols and anthocyanins) and the hydroxycinnamic acids like p-coumaric, sinapic and ferulic acids, often found in conjugation with sugar or other hydroxycinnamic acids (Olsen et al., 2009; Vallejo et al., 2004). Phenolic compounds have been intensively investigated because of their potential health-promoting effects (eg. anti-inflammatory, enzyme inhibition, antimicrobial, antiallergic, vascular and cytotoxic antitumor activity) (Crozier et al., 2009; De Pascual-Teresa et al., 2010; Vallejo et al., 2002), being mostly mentioned due to their antioxidant activity (De Pascual-Teresa et al., 2010; Podsędek, 2007).

Brassica sprouts became a popular healthy food, recommended for human diet due to the advantages of germinated seeds (low fat, rich in health-promoting phytochemicals, safe and fresh) (Hagen et al., 2009). They have high nutritive values (Bones and Rossiter, 2006) and rely on a simple and inexpensive production. Compounds like GLs can be almost 10 times higher in brassica sprouts than in mature vegetables (Fahey et al., 1997; Martinez-Villaluenga et al., 2008), showing also a predominance of aliphatic glucosinolates (Pérez-Balibrea et al., 2008), recognized for being more effective in the prevention of carcinogenesis, mutagenesis, and other forms of toxicity of electrophiles and reactive forms of oxygen than the indolic GLs, more prevalent in mature brassica vegetables (Fahey et al., 1997). The interest in sprouts has also benefit from the increased demand for fresh and safe vegetables. However, it is necessary to evaluate the changes in bioactive compounds during postharvest storage and optimize their quality, palatability and bioactivity. Several

studies have been conducted to optimize the conditions for production of sprouts, like the sprouting duration and the influence of photoperiod, in order to maximize the bioactive compounds content and their potential antioxidant activity (Vale et al., 2014). Seed-sprouts are usually harvested and immediately marketed, making the period of time between harvest and consumption and the storage conditions of great importance to avoid losses of bioactive compounds. They are usually sold in containers, and kept in domestic refrigerators until used. However, there are not much data documenting the stability of GL and phenolics in sprouts during cold storage. Furthermore, it is also important to evaluate the microbial flora since sprouts are usually consumed raw, which has led to an increase in the incidence of sprout-associated foodborne illness throughout the world (Dechet et al., 2014).

The presence of some biogenic amines has also been suggested as a supplementary criterion of freshness and quality of food (Martinez-Villaluenga et al., 2008). These compounds are organic bases of low molecular weight that can be found in plant foods since they are required in cellular metabolism and in growing tissues (Matilla, 1996; Santos, 1996). Nonetheless, their presence is also a consequence of microbial activity (Gloria et al., 2005) and putrescine, cadaverine, spermidine, spermine, histamine and tyramine were suggested as indicators of food deterioration (Paulsen et al., 1997). The relation between the biogenic amines and the microbial quality of sprouts was already reported (Frías et al., 2007; Martinez-Villaluenga et al., 2008; Simon-Sarkadi and Holzapfel, 1995), but only for few species of seed-sprouts.

The work described aimed to examine the changes in glucosinolates, phenolic compounds and biogenic amines levels, and also the evolution of the microbial population in sprouts of four varieties from *Brassica oleracea* stored under refrigeration at 4ºC.

7.2. Materials and methods

7.2.1. Reagents and Plant material

All chemicals and reagents were of analytical grade and were obtained from various commercial sources (Sigma/Aldrich and Merck). All solvents were of high-performance liquid chromatography (HPLC) grade, and all water was ultra-pure treated in a Milli-Q water purification system (Millipore, Bedford, MA, USA).

In the current study four Brassicas were selected, commonly consumed in Northern Portugal, namely Broccoli (*B. oleracea* L. var. italica Plenck, variety calabrese), Portuguese Galega (*B. oleracea* var. acephala DC), Portuguese Tronchuda cabbage (*B. oleracea* L. var. costata DC, landrace Penca da Póvoa) and red cabbage (*B. oleracea* var. capitata f. rubra). Only untreated seeds were used for sprout production. Seeds from Penca cabbage and Galega kale were acquired directly from the producers in Póvoa do Varzim (North of

Portugal); seeds from Broccoli and Red cabbage were supplied by Germisem - Sementes Lda.

7.2.2. Sprouting conditions and refrigerated storage

Seeds of the above four *B. oleracea* varieties were treated according to the method described by Vale et al. (2014). Seeds were previously sanitized with sodium hypochlorite (0.07%, v/v) for 30 minutes and washed. Then they were imbibed in water for 12 hours in darkness, at room temperature with a slight stirring. The imbibed seeds were then spread in trays containing vermiculite (10x15x4cm) and allowed to germinate in a plant growth chamber (Fitoclima 200, Aralab, Rio de Mouro, Portugal) with controlled temperature (25ºC) and photoperiod. Two types of photoperiod conditions were used in order to obtain green sprouts (GS) and white sprouts (WS). For GS the seeds were submitted to a cycle of 16 hours of light and 8 hours of darkness; whereas for WS germination was held only in the dark. Germination process was carried out in triplicate, with a germination yield over 98%. Seeds where sprouted for 9 days and watered daily. After 9 days, sprouts were cut from their root mats, divided into five lots weighing more than 10 g each and placed in polystyrene boxes (150x110x30), at 4 ºC in the dark simulating a domestic refrigerator (Binder KB115 E 3.1). A sample was collected at the harvesting time (time 0) and then boxes were removed at 2, 5, 7, 9, and 12 days for glucosinolate, polyphenols and biogenic amine analysis (see below). The entire experiment was replicated three times. Samples removed from refrigeration in each time were frozen at -80ºC, freeze-dried (Scanlaf 110-4 PRO, Lynge, Denmark) followed by powdering in a mill (Retsch ZM 200, Haan, Germany) and kept in a desiccator until analysis.

7.2.3. Analytical procedures

7.2.3.1. Glucosinolate extraction and analysis

Glucosinolates (GL) extraction was performed according to Pereira et al. (2002). Briefly, 0.2 mg of freeze dried sample was extracted with 3 mL of boiling methanol 90% (v/v) and homogenised for 2 min at 24000 rpm (Utraturrax T_{25}). After 30 seconds from start boiling, 200 µL of an internal standard solution (glucotropaeolin, 1 $mg.mL^{-1}$), was added. The homogenised sample was centrifuged for 2 min at 5000 rpm (Kubota 2100) and then re-extracted with boiling 70% (v/v) methanol. The supernatants were combined to a final volume of 10 mL. An aliquot of 2.5 mL of the extract was taken to dryness under air flow and resuspended in 2.5 mL of water. Meanwhile 0.5 mL of water was added to the Sephadex DEAE A25 column and leave to drain. Then, 2x1 mL of ressuspended extract was loaded in the column. The resin was washed twice with 1 mL of water followed by 0.5 mL of a 0.02M piridin buffer (C_5H_5N, K22146828, Merk). Finally the adsorbed GL were

desulfated by adding 75 µL of sulfatase. The reaction time was of 18 hours at 20-25ºC. Then the column was washed three times with 0.5 mL water to elute the desulfated-GL, which were collected in glass vials and preserved at -18ºC until HPLC analysis. The desulfo-GL were analyzed in an HPLC system (Gilson system, HPLC 712, Gilson). The compounds were separated in a C18 column (Spherisorb 5 µm ODS2, 250 × 4.6mm i.d., Waters). The mobile phases were composed of ultra-pure water (solvent A) and by 20% acetonitrile (solvent B). The flow rate was of 1.5 $mL \cdot min^{-1}$ and the chromatograms were recorded at 229 nm. GL peak identification and quantitative estimations were made using pure standard GL as internal standard (benzyl GL), and response factor of each GL (Aires et al., 2012). GL were expressed as 100 g of dry weight.

7.2.3.2. Polyphenol extraction and analysis

The freeze dried samples were extracted with 70% methanol. Forty milligrams of sample were extracted (in triplicate) using a standard method (Bennett et al., 2006). Concisely, two replicates were extracted with 1 mL of 70% (v/v) methanol, and the third replicate was extracted with 950µL of 70% (v/v) methanol adding also 50µL of 1mg mL^{-1} rutin (internal standard). All of the samples were heated (70ºC) for 30min with vortex mixing every 5min. After, the samples were centrifuged at 4ºC for 20 min at 17000g, and the supernatants injected in a HPLC system. HPLC analyses were performed using a Thermo Surveyor HPLC consisting of solvent degasser, quaternary pump, thermostatically controlled auto-sampler (set at 10ºC), thermostatically controlled column oven (set at 25ºC). The compounds were separated in a Phenomenex Luna C18 column (250 x 4.6mm i.d., 5µm) with a Phenomenex Security guard pre-column with a C18 cartridge. The mobile phase consisted of two solvents, solvent A, 0.1% (v/v) TFA and solvent B, acetonitrile (0.1% (v/v) TFA) with a flow rate of 1 mL min^{-1} and an injection volume of 10 µL. The identification was made comparing with external standards, their retention times and UV-vis spectra. The diode array detector recorded the spectra between 200 and 600 nm, and the chromatograms were also registered at 227, 270, 370 nm for flavonoids and phenolic acids monitorization, and 520 nm for anthocyanins.

7.2.3.3. Hygienic status and analysis of Biogenic amines

Ten grams of fresh sprouts were aseptically obtained from each sample at each sampling day and homogenized with appropriate amount of sterile 0.1% peptone water (PW) (Gelysate, BBL, USA) to make 10^{-1} dilution. The resulting homogenate was diluted serially with 10-fold PW diluents.

To determine the total mesophilic aerobic bacteria counts, appropriate serial dilutions were surface-plated on Triptic Soy Agar (TSA). Plates were incubated at 32ºC for 48 h.

Total coliforms were determined on Violet-Red Bile Agar (VRVGA) containing lactose as carbohydrate source and incubated at 37ºC (for total coliforms) or 44ºC (faecal coliforms) for 24 h. For the yeast and molds determinations, serial PW dilutions were plated with Rose-Bengal Chloramphenicol Agar (RBCA). The plates were incubated at 23 °C for 72h prior to colony enumerations. The presence of *Salmonella* was evaluated in twenty-five grams of sprouts obtained aseptically from each sample and homogenized with sterile Buffered Peptone Water (BPW). Pre-enriched cultures were then transferred to selective enrichment broth (TT) and allowed to incubate at 37ºC for 24 h. Aliquots of selectively enriched cultures were transferred to Rappaport-Vassiliadis Salmonella Enrichment Broth (RVS) and incubated for at 41,5ºC for 24 h. The inoculation was made in plates containing xylose lysine deoxycholate agar (XLD) and incubated at 37ºC for 24 h.

The extraction and HPLC analysis of biogenic amines was carried out by acid extraction, derivatization with dansyl chloride and HPLC quantification according to Frías et al. (2007). Briefly, 0.5 g of freeze-dried sample was homogenized with 10 mL of 0.1 M HCl in an Ultra-Turrax T25 homogenizer for 2 min. The homogenate was centrifuged at 12,000 rpm for 20 min at 4ºC. Supernatant was collected and the residue re-extracted under the same conditions. Combined extracts were filtered through a Whatman no. 1 filter paper and diluted to 100 mL in a volumetric flask. Dansyl-derivates were prepared with 1 mL aliquot of the diluted extract mixed with 0.5 mL of saturated $NaHCO_3$ and 1 mL of dansyl chloride (20 mg mL^{-1} in acetone). The mixture was then kept at 40ºC in darkness under agitation for 60 min. Proline solution (100 mg ml^{-1}) was used to remove residual dansyl chloride by vortexing 200µL of proline for 1 min. The mixture was left to react at room temperature in darkness for 15 min. Two final extractions were performed with 1 ml of diethyl ether (SDS) and the combined extracts were dried under nitrogen flow. The residue was dissolved in 0.5 ml of acetonitrile, and then filtered through a 0.45µm PVDF Millipore filter before injection.

The chromatographic system was a Jasco equipment consisting of solvent degasser, quaternary gradient pump and auto-sampler thermostatically controlled. A volume of 20 µL was injected onto a C18 Kromasil (250x4.6 mm i.d., 5µm); equipped with a C18 guard column both thermostatted at 30ºC. The mobile phases consisted of ultrapure water (solvent A) and acetonitrile (solvent B). The elution gradient was held at 65% of B for 1 min, ramped at 80% (10 min), 90% (12 min), and 100% of B (16 min) and held until the end of the run (23 min) with a flow rate of 0.8 mL/min. The chromatograms were recorded at 254nm by a diode-array detector.

A stock standard aqueous solution of different amines was prepared by adding an accurately weighed amount of each standard (ca. 80 mg) to a 25 mL volumetric flask.

Standards were derivatized as described for the samples. Calibration curves were obtained for standard amines and Pearson correlation (r) was always above 0.996.

7.2.4. Statistical analysis

Data obtained from the study were presented as mean ± standard deviation and the differences between samples, growth conditions and shelf life under refrigeration were tested by one-way ANOVA followed by post-hoc Tukey comparison tests, using the SPSS 15.0 software (SPSS Inc., Chicago, Illinois, EUA) for Windows. Statistical significance was defined for $p < 0.05$.

7.3. Results and discussion

Sprouts are known for being a fresh and convenient but also very perishable food which can contribute to the overall diet intake of vegetables. As mentioned earlier, their bioactive compounds are an added value, influencing food choice due to an increased perception of the health benefits of including fresh and nutritious vegetables in the diet (Poiroux-Gonord et al., 2010). As ready-to-eat products, sprouts are stored and commercialized under refrigerated conditions, which are likely to influence the overall quality and composition (Vallejo et al., 2003a). This work focused on the glucosinolate and phenolic evolution and microbial security of four varieties of refrigerated sprouts, knowing already from a previous work (Vale et al., 2014) that their bioactive compounds content can be influenced by genetics and by different sprouting conditions. Their microbial load was monitored throughout storage to establish the shelf life of these sprouts.

7.3.1. Variation of total and individual glucosinolates content

All the Brassica sprouts showed a significant ($p<0.05$) decrease of their total GLs level over of storage the 12 days at 4ºC (see Figure 7.1). A decrease of total and individual GLs in vegetables stored under refrigeration (4-8 ºC) for 7 days was also reported by Song and Thornalley (2007). However, Force et al. (2007) did not found significant loss of GLs in different brassica sprouts during 7 days of storage at 4 ºC, as it was seen in the four *B. oleraceae* varieties studied. The sprouts from Galega kale were the ones that showed the biggest loss of GLs (losses of 90%) during the twelve days of storage, followed by Penca cabbage (50%) and red cabbage (39%) sprouts. Broccoli sprouts showed the smaller differences between their initial (day 0) and final (day 12) levels. In the analyzed sprouts, the loss of glucosinolates begun in the first 5 to 7 days of storage, being more intense after that period. Broccoli green sprouts (GS) showed a different behavior, especially during the first 5 days of storage, when their GLs content increased 123%. Other samples also showed an increase of GLs after one week of storage, namely Galega kale GS (at the 7th day),

Penca cabbage (also at the 7th day) and red cabbage sprouts (at the 9th day of storage for GS and at 7th for WS), decreasing in the following days (see figure 7.1). This different behaviors between each *B. oleracea* variety revealed a clear influence of the genetics on the glucosinolate stability during refrigerated storage, as it was also seen in the work of Force et al. (2007).

The light exposure during sprouting had also a clear influence on the GLs content. Sprouts from Penca cabbage growth under darkness (WS) showed a two times higher content of GL than the correspondent GS, while in sprouts from Red cabbage variety occurred the opposite behavior with WS having about the half of the GL content found in the GS. In the other two varieties the initial differences between GS and WS was less pronounced. Regarding the evolution of the GL content during storage, sprouts grown under darkness showed a more stable content during the first days of storage than the correspondent GS. This tendency was more evident in the Galega kale and Penca cabbage GS sprouts, than in the Broccoli or Red cabbage that showed a more stable GL content during the all storage period.

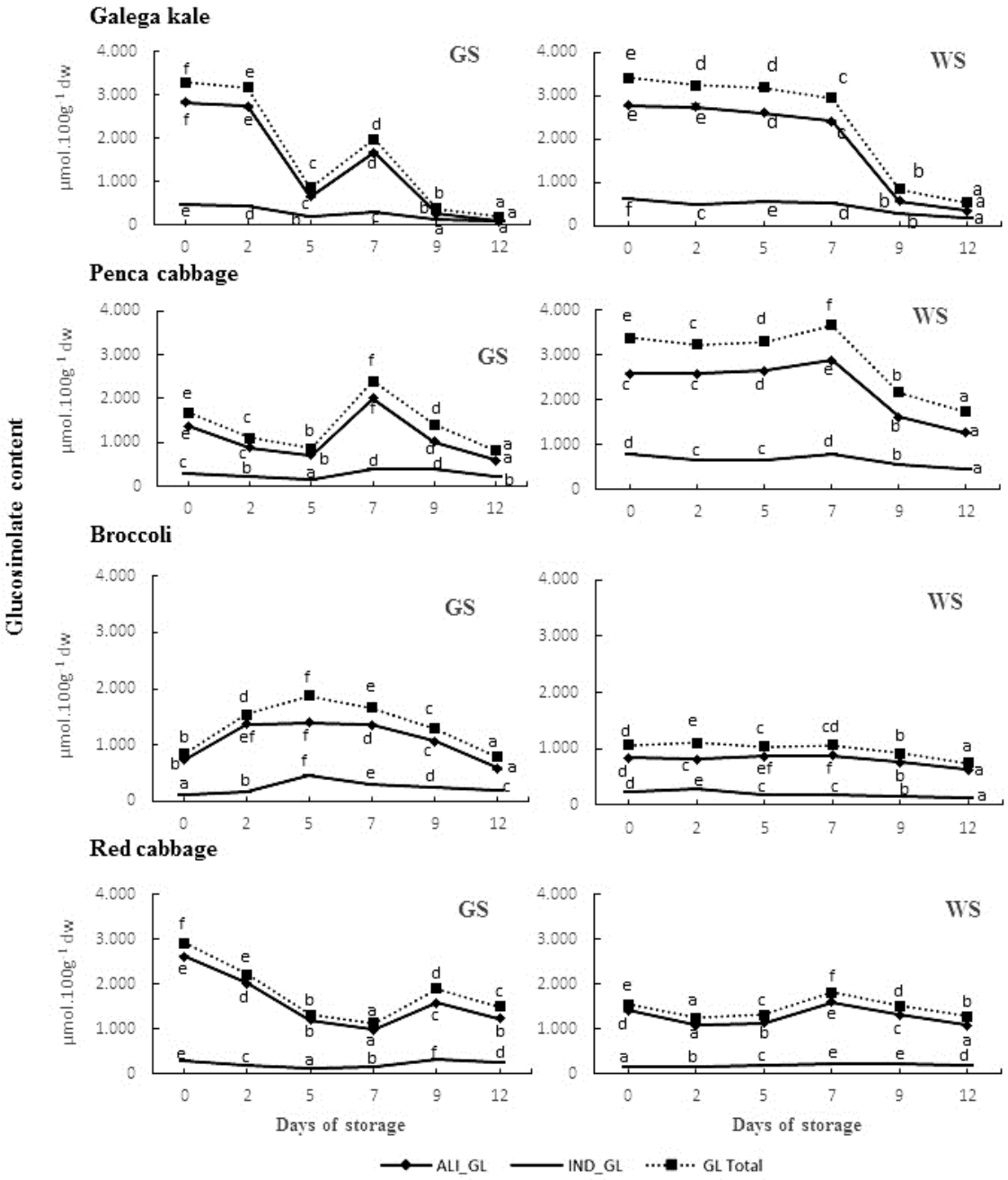


Figure 7.1 Evolution of the aliphatic (ALI_GL), indole (IND_GL) and Total (GL Total) glucosinolates content (µmol.100g-1 dw) of *Brassica* sprouts, stored at 4 °C grown under light (GS) and darkness (WS) condition. Different letters in the same line mean significantly differences in GL content during shelf life ($p < 0.05$).

Regarding the glucosinolate profile of the sprouts, all samples studied showed a prevalence of aliphatic glucosinolates over the indole-GL (see Figure 7.1 and Table 7.1). Thus, the overall variation of the Total GL content discussed above, resulted principally from the variation occurred in the aliphatic-GL content of the sprouts (see Figure 7.1),

showing the levels of indole-GL a smaller variation during the refrigerated storage of the sprouts. The highest content of aliphatic GLs was recorded in Galega kale GS (2825 µg.100g^{-1} dw at harvest) and in the Penca cabbage WS (2876 µg.100g^{-1} dw, at the 7th day of storage). In relation to indole GL the highest concentrations were observed in the broccoli GS (476 µg.100g^{-1} dw, at the 5th day of storage) and in the Penca cabbage WS (795 µg.100g^{-1} dw, at harvest). The relative contribution of aliphatic GLs to total GL varied from 88% in red cabbage sprouts, to 79% in Penca cabbage. The profile variation of each individual GLs during storage of sprouts is presented in Table 7.1. Sinigrin was the main GL present in Galega kale and Penca cabbage sprouts, followed by 4-methoxyglucobrassicin and glucoiberin in Galega kale and in the reverse order in Penca cabbage sprouts. These three glucosinolates represent more than 90% of Galega kale and more than 80% of Penca cabbage glucosinolate profile. In this sense, the evolution of the glucosinolate content described for these two varieties is mainly caused by the changes of these individual compounds. Galega kale sprouts lost 97% and a 90% of the sinigrin content in GS and WS, respectively, during the 12 days of storage, being the content more stable in WS during the first 7 days. A similar behavior was recorded for 4-methoxyglucobrassicin (75 and 59% loss in GS and WS, respectively) and glucoiberin (94% and 75% loss in GS and WS, respectively) (see Table 7.1). In Penca sprouts, these three major glucosinates had the same evolution described for Galega kale, showing however a slight inferior percentage of loss during the 12 days of storage.

Table 7.1 Variations in GL profile (µmol.100g-1 dw) during the shelf life of sprouts from four varieties of Brassica produced under light (GS) and darkness (WS) condition. Different letters mean significantly differences in GL content during storage ($p < 0.05$).

	GS						WS					
Days	0	2	5	7	9	12	0	2	5	7	9	12
Glucosinolates	**Galega kale**											
Glucoiberin	388.07±5.0^{d}	432.47±1.9^{e}	163.83±0.7^{c}	590.36±1.5^{f}	55.25±2.0^{b}	23.2±1.1^{a}	537.46±4.7^{e}	473.27±3.7^{d}	523.01±3.4^{e}	440.2±3.0^{c}	195.93±0.2^{b}	135.19±0.8^{a}
Progoitrin	198.95±2.8^{e}	175.47±1.8^{d}	35.95±2.7^{b}	76.82±1.8^{c}	17.6±1.2^{a}	nd	79.41±1.2^{b}	16.94±3.2^{a}	nd	nd	nd	nd
Glucoraphanin	nd	nd	nd	52.93±1.9	nd	nd	48.52±1.9^{c}	23.9±1.6^{b}	13.45±1.3^{a}	nd	nd	nd
Sinigrin	2238.4±11.4^{f}	2124.1±17.8^{e}	443.07±2.9^{c}	952.32±2.8^{d}	178.13±2.3^{b}	60.52±0.6^{a}	2115.03±12^{d}	2234.35±18.12	079.51±1.2^{d}	1976.5±12^{c}	368.71±5.2^{b}	219.79±1.5^{a}
4-hydroxyglucobrassicin	nd	nd	nd	30.96±2.7	nd	nd	160.9±1.4^{e}	135.9±1.8^{c}	147.68±1.6^{d}	150.45±3.2d	65.5±1.5^{b}	35.77±1.7^{a}
Glucobrassicin	72.96±2.4^{e}	57.17±0.4^{d}	20.89±1.8^{b}	30.16±1.0^{c}	7.17±0.4^{a}	nd	87.43±2.9^{d}	76.97±1.9^{d}	63.2±1.9^{c}	44.87±2.6b	16.52±0.6^{a}	13.79±0.8^{a}
4-methoxyglucobrassici	362.8±1.2^{d}	348.66±11.7^{d}	173.43±0.7^{b}	243.12±2.2^{c}	107.66±3.4^{a}	91.32±2.9^{a}	309.63±2.8^{d}	240.57±2.3^{c}	316.29±3.4^{d}	305.06±2.6^{d}	191.82±3.8^{b}	126.74±0.4^{a}
Neoglucobrassicin	31.3±1.5^{c}	25.13±1.2^{b}	8.94±0.02^{a}	nd	nd	nd	74.11±4.6^{d}	47.33±0.8^{c}	52.13±1.8^{c}	35.69±0.9^{b}	9.36±0.5^{a}	nd
	Penca cabbage											
Glucoiberin	337.68±1.2^{e}	213.83±1.4^{b}	223.32±2.9^{b}	543.24±1.7^{e}	268.26±1.5^{c}	113.54±4.2^{a}	915.5±1.0^{c}	929.45±2.0^{c}	962.12±3.8^{d}	1115.7±3.1^{e}	660.73±3.7^{b}	552.2±3.2^{a}
Progoitrin	73.63±2.9^{c}	52.16±1.9^{b}	40.28±1.0^{a}	108.48±2.1^{d}	54.89±2.6^{b}	48.8±0.3^{ab}	108.95±5.0^{c}	131.22±2.1^{d}	131.5±0.6^{d}	106.98±2.6^{c}	72.96±0.9^{b}	58.59±2.3^{a}
Glucoraphanin	58.57±2.8^{d}	45.49±1.4^{bc}	36.13±2.3^{b}	83.54±1.3^{e}	46.97±3.1^{c}	23.61±1.0^{a}	165.63±2.9^{b}	149.5±1.7^{d}	157.23±1.5^{e}	194.91±2.4^{d}	125.48±2.2^{c}	95.49±0.8^{a}
Sinigrin	902.09±1.2^{d}	572.18±2.2^{b}	413.56±0.7^{a}	1277.4±2.8^{e}	650.42±3.9^{c}	405.54±1.5^{a}	1388.56±3.7^{d}	1372.28±2.0^{c}	1385.41±2.6^{d}	1458.84±1.2^{e}	748.95±0.3^{b}	559.8±3.4^{a}
4-hydroxyglucobrassicin	42.44±2.2^{c}	19.5±1.1^{b}	6.049±0.5^{a}	nd	nd	nd	179.98±2.7^{c}	151.54±3.6^{b}	156.29±1.7^{b}	195.97±2.6^{d}	156.55±1.8^{b}	133.96±1.4^{a}
Glucobrassicin	82.25±3.1^{f}	58.63±0.7^{d}	26.83±1.4^{b}	68.31±1.7^{e}	44.1±1.2^{c}	17.98±0.9^{a}	43.94±1.1^{c}	l0.7574±0.4^{bi}	55.47±1.4^{d}	71.8±2.1^{e}	36.22±0.6^{b}	27.08±1.9^{a}
4-methoxyglucobrassici	172.92±1.2^{c}	147.06±3.5^{b}	124.67±1.2^{a}	322.95±2.5^{e}	337.39±1.7^{f}	198.63±1.0^{d}	476.35±2.5^{e}	380.52±2.6^{c}	369.42±4.5^{c}	444.66±2.2^{d}	347.28±3.4^{b}	290.95±2.9^{a}
Neoglucobrassicin	14.06±1.0	nd	nd	nd	nd	nd	94.56±2.0^{d}	67.86±0.8^{bc}	69.93±1.1^{c}	64.21±0.7^{b}	20.26±1.1^{a}	15.66±0.61^{a}
	Broccoli											
Glucoiberin	58.71±1.2^{cd}	67.0±0.9^{d}	49.19±0.9^{ab}	54.03±1.2^{bc}	43.02±0.2^{a}	41.83±0.4^{a}	199.5±1.1^{c}	187.67±1.4^{b}	221.96±1.0^{d}	236.5±1.1^{e}	203.63±2.8^{c}	154.7±0.4^{a}
Progoitrin	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Glucoraphanin	672.97±1.7^{b}	1310.48±4.2^{d}	1351.63±4.7^{e}	1309.7±5.4^{d}	1017.47±2.9^{c}	542.34±2.6^{a}	640.7±2.1^{c}	626.74±0.7^{c}	640.14±3.4^{c}	642.36±6.5^{c}	553.77±4.7^{c}	466.23±2.2^{a}
Sinigrin	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
4-hydroxyglucobrassicin	nd	nd	37.5±2.3	nd	nd	nd	41.05±0.1^{c}	59.4±3.0^{d}	31.58±1.5^{b}	26.56±01.5^{ab}	22.4±0.7^{b}	13.96±0.5^{a}
Glucobrassicin	13.77±1.3^{a}	27.64±0.6^{b}	54.9±0.8^{d}	34.47±2.1^{c}	32.54±1.3^{bc}	nd	13.31±0.3^{a}	15.7±0.6^{ab}	19.91±0.5^{c}	19.41±0.7^{c}	16.23±0.7^{b}	14.12±0.2^{ab}
4-methoxyglucobrassici	94.93±2.7^{a}	139.28±0.5^{b}	383.07±2.7^{e}	271.5±1.4^{d}	205.64±3.1^{c}	200.22±4.1^{c}	157.68±0.5^{d}	199.48±0.6^{e}	106.31±0.7^{b}	113.96±1.3^{c}	113.24±1.6^{c}	95.16±1.6^{a}
Neoglucobrassicin	nd	nd	nd	nd	nd	nd	15.79±0.5^{b}	16.94±0.3^{b}	22.57±0.4^{c}	20.66±0.6^{c}	14.9±0.3^{b}	5.8±0.2^{a}
	Red cabbage											
Glucoiberin	527.76±1.8^{f}	356.15±2.8^{e}	252.31±2.7^{b}	188.48±1.7^{a}	338.13±1.1^{d}	304.84±1.8^{c}	258.18±2.2^{b}	219.71±0.7^{a}	214.34±1.0^{a}	313.94±1.7^{c}	252.23±3.6^{b}	215.8±0.6^{a}
Progoitrin	706.93±3.1^{e}	546.47±2.5^{d}	276.43±2.5^{b}	225.07±2.7^{a}	429.91±3.5^{c}	285.21±1.2^{b}	372.85±2.7e	252.79±1.3a	319.68±0.4c	358.96±0.4d	323.71±1.5c	264.38±1.0b
Glucoraphanin	984.47±0.6^{e}	773.43±2.8^{d}	448.39±1.3^{b}	411.3±4.2^{a}	611.29±4.2^{c}	456.87±2.8^{b}	478.32±2.6^{d}	391.03±3.3^{b}	369.76±2.0^{a}	604.64±0.7^{e}	467.62±1.0^{c}	390.49±1.8^{b}
Sinigrin	397.71±2.5e	334.28±1.7d	221.93±0.5c	150.43±4.0a	192.54±2.9b	184.09±1.6b	300.15±2.2^{e}	224.84±0.9^{b}	232.29±0.3^{c}	317.85±1.7^{f}	263.28±1.7^{d}	215.59±0.6^{a}
4-hydroxyglucobrassicin	nd	nd	nd	17.05±1.4^{a}	29.35±1.5^{c}	23.86±1.7^{b}	58.8±1.1^{c}	45.29±1.0^{ab}	45.49±0.9^{ab}	48.19±0.8^{b}	48.44±1.0^{b}	42.22±0.4^{a}
Glucobrassicin	28.49±1.6^{d}	25.24±1.3^{cd}	11.48±0.5^{a}	8.06±0.5^{a}	19.8±0.6^{bc}	17.77±0.6^{b}	11.57±0.4^{a}	19.67±0.5^{c}	18.18±0.1^{c}	18.91±0.1^{c}	19.69±0.3^{c}	15.36±0.3^{b}
4-methoxyglucobrassici	258.5±1.8^{e}	178.96±1.9^{c}	109.08±2.6^{a}	119.74±1.5^{b}	275.2±0.6^{f}	228.41±0.7^{d}	64.36±2.0^{a}	94.66±2.3^{b}	102.43±0.7^{c}	142.39±0.4^{e}	141.47±0.7^{e}	128.95±0.9^{d}
Neoglucobrassicin	nd	nd	nd	9.31±0.5	nd	nd	9.49±0.4^{a}	nd	11.21±0.6^{b}	nd	nd	nd

Glucoraphanin was main GL found in broccoli (representing 77% of total GL content in GS and 60% in WS), which is in agreement with previous reports (Fahey et al., 1997; Force et al., 2007; Pereira et al., 2002; West et al., 2002). Although with a smaller predominance (around 30 to 34%), glucoraphanin was also the main GL found in red cabbage sprouts, also followed by glucoiberin and 4-methoxyglucobrassicin. In these later brassica varieties, the differences between the initial content of these glucosinolates and the one found after 12 days of storage was lower than the recorded for Penca cabbage and Galega kale sprouts.

Several break-down products of aliphatic GLs are known for reduce the risk of cancer, especially sulforaphane that derives from glucoraphanin and sinigrin (Cieślik et al., 2007), which were some of the main compounds found in the studied sprouts as stated earlier. However, to maximize the health benefits that could arise from sprouts consumption, broccoli, Penca cabbage and Galega kale sprouts should be consumed within the first 7 days after harvest. During that period GS of broccoli and red cabbage and WS of Galega kale and Penca cabbage showed higher levels of these aliphatic GL, reveling that the sprouting conditions must be adequate to each variety to potentiate their GL content production (see Table 7.1).

7.3.2. Variation in contents of phenolic compounds

Owing to the fact that phenolic compounds exist mainly in nature in conjugated form with sugars and organic acid moieties, a complete identification of the specific compounds present in each sprout profile was not possible with the data obtained with DAD analysis and comparison with the available external standards. The phenolic compounds present in the brassica sprouts were classified accordingly to their UV-Vis spectra into the different classes of phenolic compounds. The hydroxycinnamic acids that exhibit an absorbance maximum around 320-330 nm (Carazzone et al., 2013) were the main compounds found in the majority of the brassica sprouts extracts. The presence of derivatives of sinapic acid was described in Penca cabbage seeds and sprouts and also in other *B. oleracea* sprouts varieties (Ferreres et al., 2007; Pajak et al., 2014; Sousa et al., 2007), being the importance of this phenolic group in plant organs of *B. oleracea* varieties reported in several works (Ferreres et al., 2006; Vallejo et al., 2004; Vallejo et al., 2003). Thus, the hydroxycinnamic acids derivatives were quantified using the sinapic acid standard to construct an external calibration curve, being the results presented in Figure 7.2.

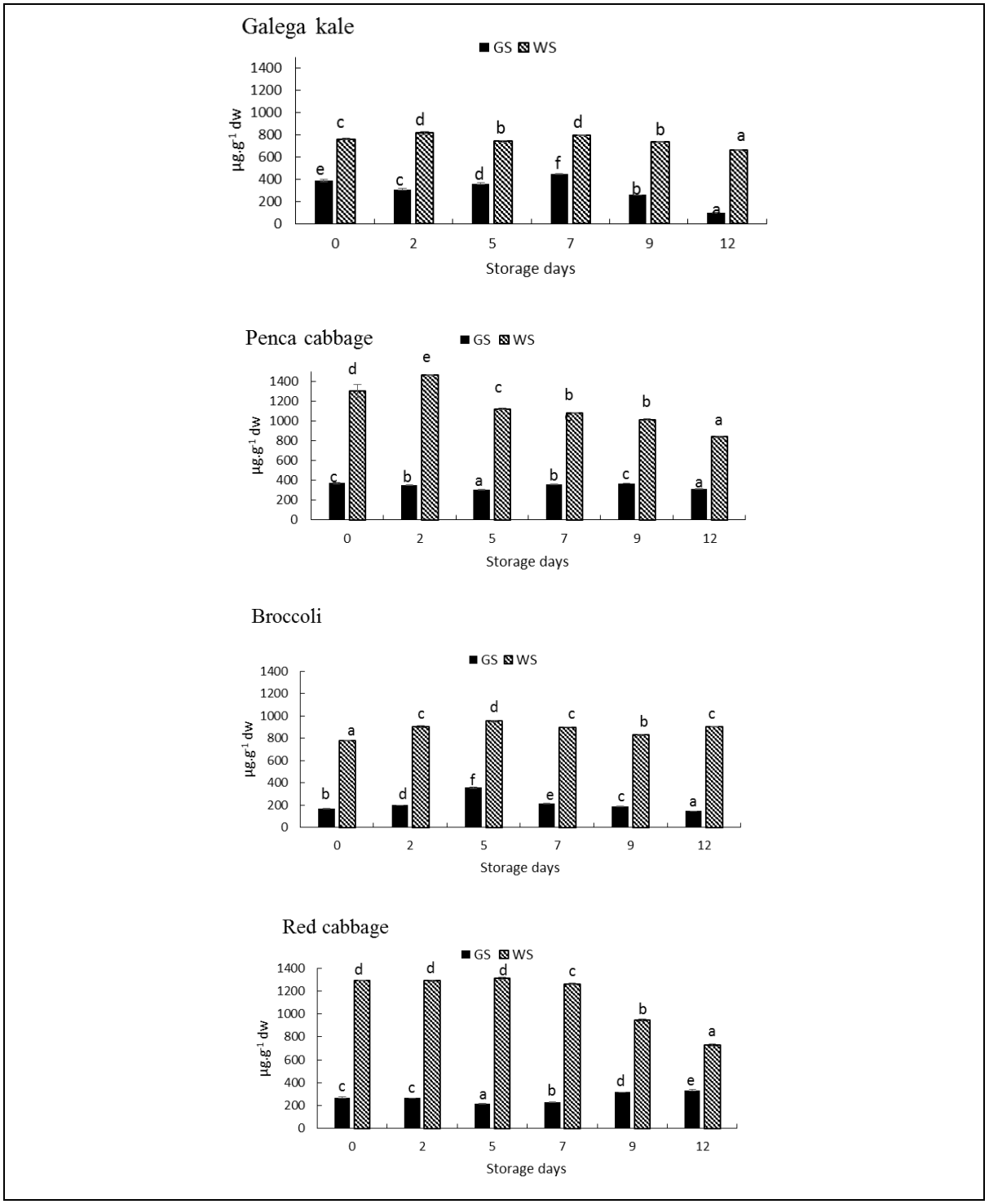


Figure 7.2 Changes in hydroxycinnamic acid concentration (µg.g-1 dw) during storage at 4 °C of sprouts from four brassica varieties, grown under light (GS) and darkness (WS) condition. The data

represent the mean of three replicates. Mean values in the same type of column sharing the same letter are not significantly different ($p < 0.05$).

The hidroxycinnamic acids content was significantly influenced by brassica variety, photoperiod and time of storage ($p< 0.05$). The sprouts produced under dark conditions (WS) showed the highest concentration of phenolics, with the Red and Penca cabbages showing the highest values, 1138 $\mu g.g^{-1}$ and 1137 $\mu g.g^{-1}$, respectively.

The majority of the samples analyzed showed significant losses ($p<0.05$) at the 12th of storage, as a result of tissue degradation. In WS, the highest losses were registered in Penca cabbage and Red cabbage (35% and 44 %, between their initial (day 0) and final content (day 12), respectively). Regarding the evolution of the phenolic content in sprouts growth under light and darkness cycles (GS), the other two varieties, Galega kale and broccoli, had the highest percentages of losses (78%, between the 7th and the 12th day, and 60% of losses between the 5th and the 12th day of storage, respectively). High loss rates of hydroxycinnamic acids derivatives were reported by (Vallejo et al., 2003a) in broccoli inflorescences during transport and distribution period (cold storage at 1^{o}C) and during retail sale period (15^{o}C). In sprouts, the losses seen at the final time of refrigerated storage could be due to the decay of vegetable tissues due to breakdown of the cellular structure caused by senescence process. However, some exceptions were observed, namely in Red cabbage GS and in the Broccoli WS, where a slight increase in the hydroxycinnamic acids content was found after the 12 days of storage. A small increase of the initial content of hydroxycinnamic acids was registered in almost all samples at the 2nd, 5th or 7th day of storage (see Figure 7.2). These situation may results from the biosynthesis of new phenolic compounds that can be triggered as a reaction to stress in the first days after harvesting and during refrigerated storage of vegetables (Starzyńska et al., 2003).

In the Red cabbage extracts, besides the hydroxycinnamic acids, another class of phenolic compounds was also identified. In these sprouts, 4 different anthocyanins, with a maximum of absorbance at 520 nm were identified (see Figure 7.3). The anthocyanins were identified as derivatives of malvidin, peonidin and cyanidin which were also in accordance to the results presented by Moreno et al. (2010) and Scalzo et al. (2008). Two derivatives of malvidin were identified, malvidin-3-galactoside and malvidin-3-glucoside, representing 77% and 84% of total phenolics in the GS and in the WS, respectively. The photoperiod used for sprout production and the storage duration had a significant effect ($p<0.05$) on the concentration of anthocyanins in the red cabbage sprouts. Generally, their concentration increased along the storage period, with the exception of malvidin-3-galactoside in the WS that showed a 35% decrease between harvest and the 12th day of storage. The increase of anthocyanins content was more pronounced in GS than in WS.

Peonidin-3-glucoside increased 39% (GS) and 10% (WS), while malvidin-3-glucoside increased 26% (GS) and 11% (WS) during storage. The flavonoids are described as more stable compounds than the hydroxycinnamic acids during refrigerated storage of vegetables (Bergquist et al., 2005; Santos et al., 2014; van der Sluis et al., 2001), which also corroborated by the smaller differences found the antocyanins evolution during storage, when compared to the higher losses found for the hydroxycinnamic content.

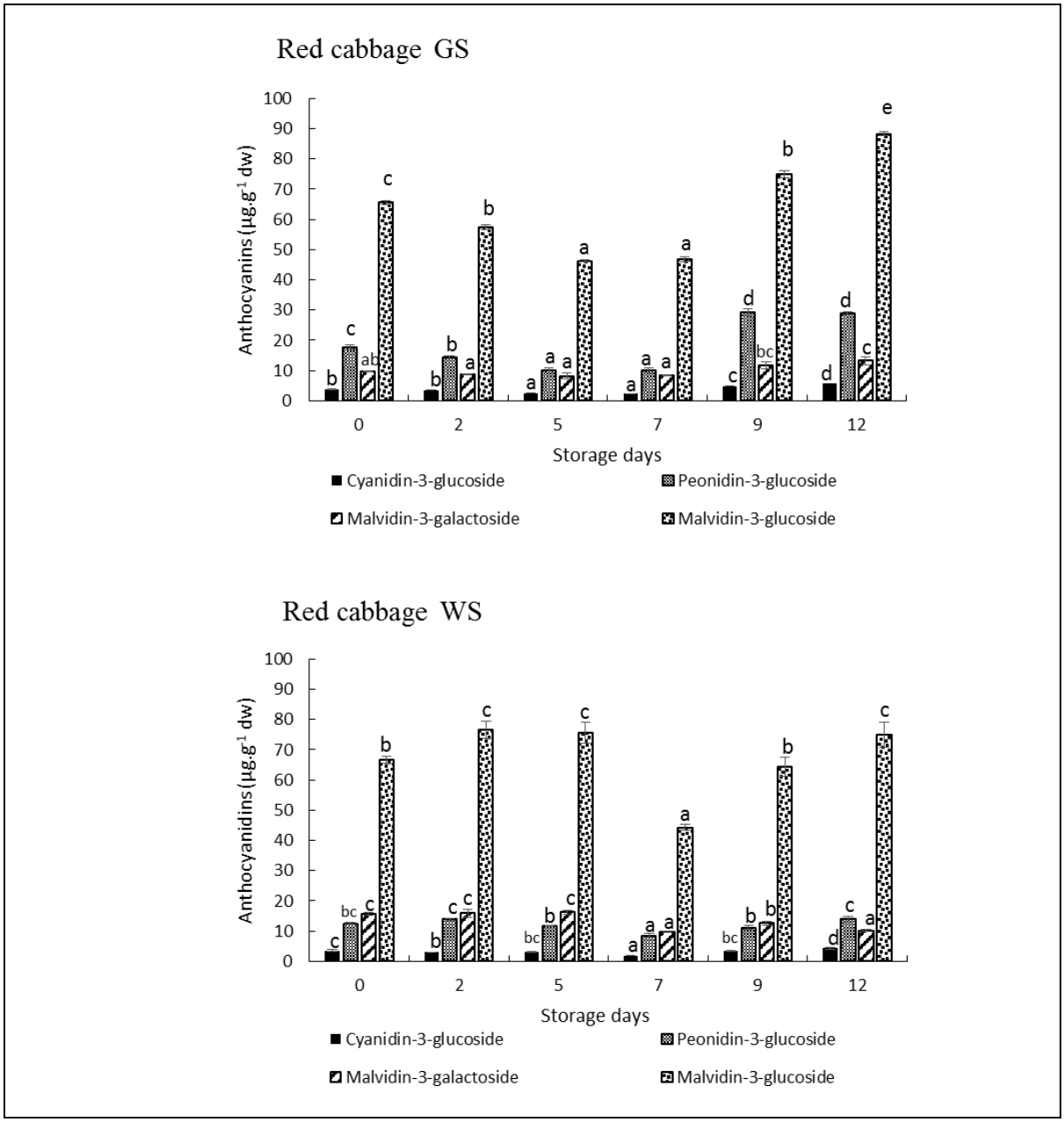


Figure 7.3 Anthocyanins content of red cabbage sprouts, grown under light (GS) and darkness (WS) conditions, during storage at 4°C for 12 days. The data represent the mean of three replicates. Mean values in the same type of column sharing the same letter are not significantly different ($p < 0.05$).

7.3.3. Microbial counts and Biogenic amines

The results obtained regarding the microbial population of the brassica sprouts during storage is presented in Table 7.2. All sprouts were evaluated for mesophilic bacteria, total and fecal coliforms, yeasts and molds and pathogenic microorganisms (*salmonella*). No contamination by pathogenic microorganisms was found in all samples analyzed grown under the different conditions.

Table 7.2 Microbial population in sprouts from four varieties of *Brassica oleracea* stored under refrigeration (4ºC) for twelve days.

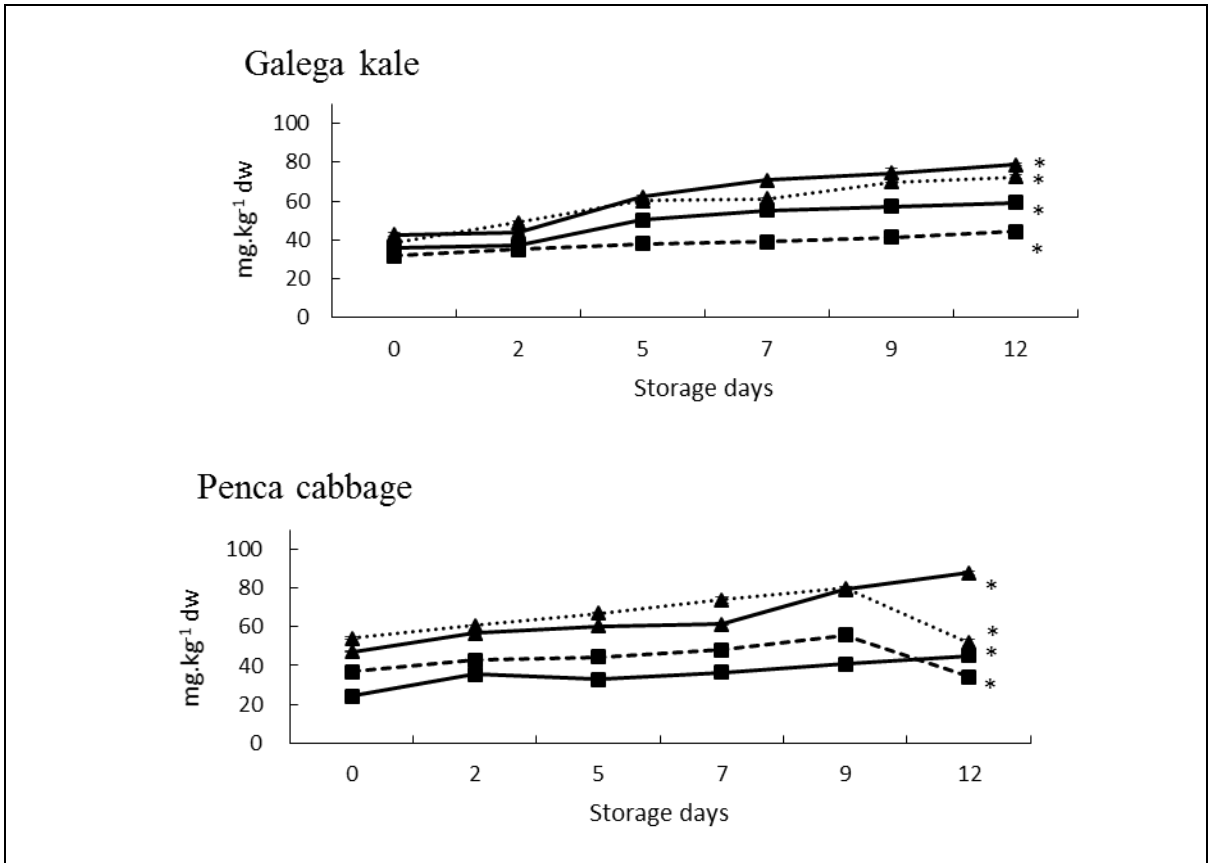
Counts (log 10 cfu.g^{-1} fresh sprout)	Storage (days)					
	0	2	5	7	9	12
			Galega kale			
Mesophilic bacteria	6.6±0.05^{a}	6.9±0.0^{b}	8.38±0.09^{c}	9.75±0.02^{d}	10.45±0.02^{e}	10.95±0.02^{f}
Total coliforms	5.46±0.01^{a}	5.84±0.0^{b}	6.23±0.02^{c}	6.58±0.02^{d}	7.7±0.03^{e}	9.4±0.0^{f}
Yeasts and molds	2.94±0.15^{a}	3.69±0.0^{b}	4.63±0.02^{c}	5.76±0.01^{d}	7.43±0.01^{e}	8.82±0.01^{f}
			Penca cabbage			
Mesophilic bacteria	6.89±0.03^{a}	7.93±0.0^{b}	8.67±0.01^{c}	10.84±0.01^{d}	10.95±0.0^{e}	10.95±^{e}
Total coliforms	4.08±0.01^{a}	5.57±0.01^{b}	6.62±0.00^{c}	8.75±0.02^{d}	9.73±0.01^{e}	9.95±0.0^{f}
Yeasts and molds	3.68±0.03^{a}	3.82±0.01^{b}	4.03±0.04^{c}	5.2±0.04^{d}	5.85±0.01^{e}	7.76±0.02^{f}
			Broccoli			
Mesophilic bacteria	7.57±0.06^{a}	7.79±0.04^{b}	8.85±0.03^{c}	9.86±0.01^{d}	9.95±0.01^{e}	9.95±0.0^{e}
Total coliforms	4.21±0.01^{a}	4.52±0.01^{b}	5.67±0.01^{c}	8.76±0.01^{d}	9.89±0.0^{e}	9.95±0.01^{e}
Yeasts and molds	2.58±0.1^{a}	2.84±0.01^{b}	3.25±0.07^{c}	3.43±0.05^{c}	5.79±0.01^{d}	8.94±0.00^{e}
			Red cabbage			
Mesophilic bacteria	6.06±0.06^{a}	6.39±0.04^{b}	7.72±0.01^{c}	9.18±0.01^{d}	10.77±0.01^{e}	10.95±0.01^{f}
Total coliforms	3.72±0.05^{a}	4.04±0.02^{b}	4.61±0.03^{c}	6.59±0.04^{d}	7.79±0.1^{e}	8.83±0.0^{f}
Yeasts and molds	3.38±0.05^{a}	3.53±0.08^{a}	4.37±0.02^{b}	5.89±0.01^{c}	8.26±0.07^{d}	9.72±0.01^{e}

Results are expressed as mean value ± SD of three repetitions. Different superscript in the same line for each variety means significant difference (P≤ 0.05)

A significant increase ($p<0.05$) of microbial population during storage was observed. At harvest (storage 0) the average initial total aerobic mesophilic bacteria was of 6.78 log 10 cfu.g^{-1}, rising 58 % up to 10.7 log 10 cfu.g^{-1} after twelve days of storage. The coliform population increased 121% from 4.3 to 9.5 log 10 cfu.g^{-1} and the population of yeasts and molds increased 184% from 3.1 to 8.8 log 10 cfu.g^{-1}. The levels of microbial contamination found in brassica sprouts, for mesophilic microorganisms and total coliforms, was in agreement with the usual counts detected in minimally processed germinated seeds such as alfalfa, bean, lupin, fenugreek or onion (Gandhi and Matthews, 2003; Lang et al., 2000; Martinez-Villaluenga et al., 2006; Prokopowich and Blank, 1991) and with counts observed in broccoli and radish sprouts (Martinez-Villaluenga et al., 2008).

The presence of biogenic amines can be expected in all foods that contain proteins or free amino acids, and which are exposed to conditions enabling biochemical and/or

microbial activity (Frías et al., 2007). The presence of biogenic amines is considered an indicator of the hygienic conditions of food. Their monitoring is also of utmost importance since an excessive consumption of biogenic amines can cause severe health problems (Bardócz, 1995). In the studied brassica sprouts the biogenic amines found during the refrigerated storage were ma putrescin and cadaverin (Figure 7.4), which have been produced due to high decarboxylase activity as a result of bacterial microorganisms activity (Simon-Sarkadi and Holzapfel, 1995). The differences in the biogenic amines concentration can be mainly explained by differences between brassica varieties and by the duration of storage ($p<0.05$) since the photoperiod used during sprouting had no significant effect in the levels of these compounds ($p> 0.05$).



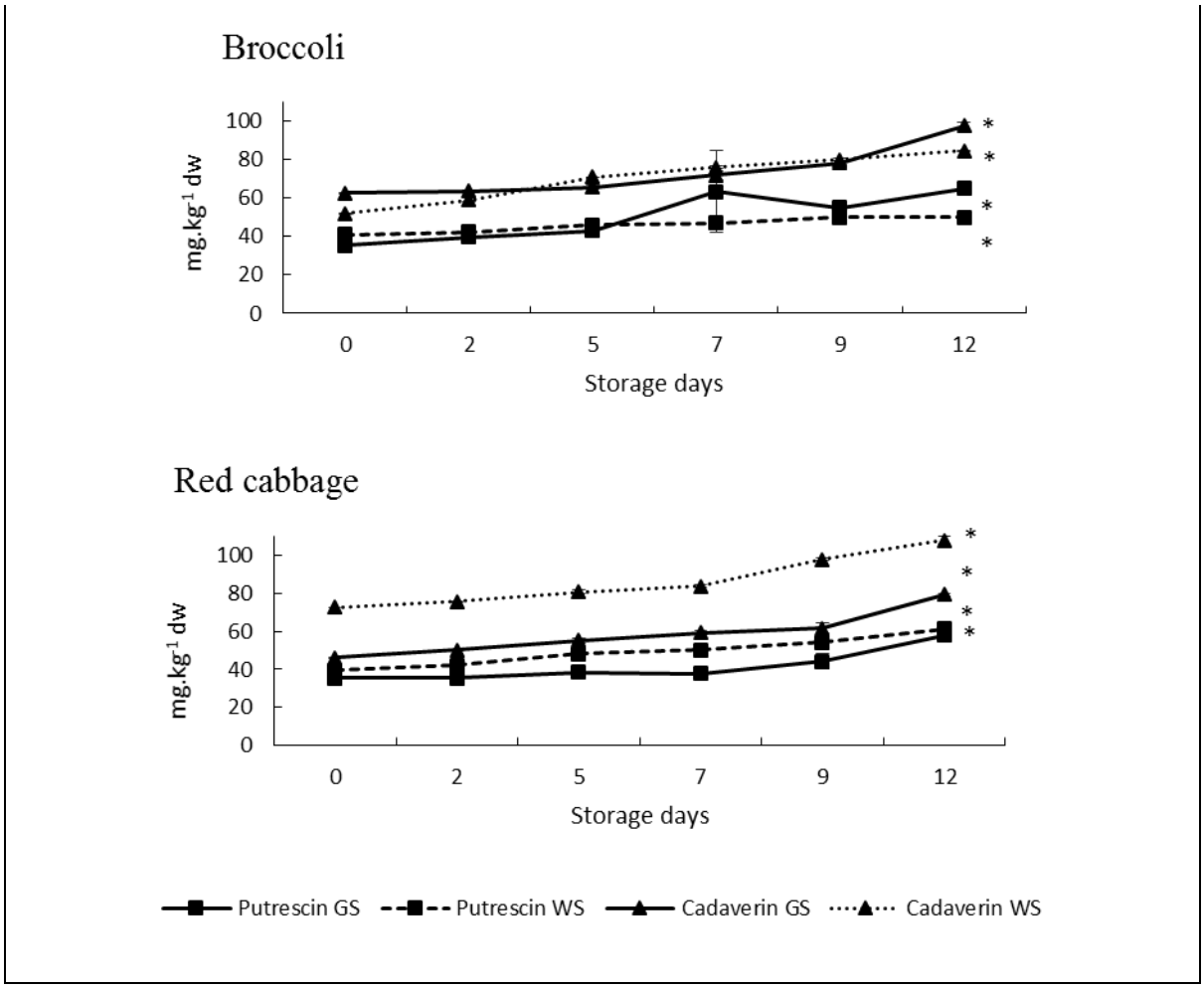


Figure 7.4 Biogenic amines content in four varieties of *Brassica* sprouts grown under light (GS) and darkness (WS) during storage at 4 °C for 12 days. Data are means of three replicates. Least significant differences ($p<0.05$) are indicated by *.

In all brassica sprouts analyzed, cadaverin was the main biogenic amine quantified, with an average concentration of 67.4 $mg.kg^{-1}$ dw whilst putrescin concentration was of 44.0 $mg.kg^{-1}$ dw. Their concentration increased during storage and the highest concentrations were observed at day 12, except in Penca cabbage WS, where putrescin and cadaverin increased (68% and 46%, respectively) until day 9 decreasing thereafter. In Broccoli and red cabbage sprouts the concentration of cadaverin increased 37% and putrescin increased 32% and 37%, respectively. The biogenic amines results found in these brassica sprouts were in agreement to those presented by Martinez-Villaluenga et al. (2008) that referred cadaverinn and putrescin as the main biogenic amines present in broccoli sprouts. However, the total biogenic amines found in their sprouts with 5 days of germination was

higher content than the total biogenic amines showed by brassica sprouts at the end of the refrigerated storage.

The allowed limits for biogenic amines are established only for the amines with higher toxicity effects, like histamine and tyramine. For putrescine and cadaverine, there is not enough data to identify concentrations which directly cause acute adverse health effects and/or potentiate the toxic effects of histamine and other biogenic amines (EFSA, 2011). According to the EFSA (2011) no adverse health effects have been observed in healthy volunteers exposed to 25 to 50 mg of histamine per person per meal and 600 mg of tyramine per person per meal. Based on these values and taking the same allowed limits for cadaverine and putrescine in sprouts, the results of the present work indicate that none of the biogenic amine levels, in any of the sprouts studied, represent a risk for healthy consumers. The maximum level of intake of biogenic amines encountered in these brassica sprouts, assuming a consumption of 100g of sprouts in a meal, was of 10.8 mg of cadaverin in the consumption of red cabbage sprouts stored at 4ºC for 12 days.

7.4. Conclusions

A refrigerated storage for more than 7 days revealed a significant decrease of the levels of GLs in sprouts. Further storage, induces a significant decrease of glucoraphanin and sinigrin that are the two compounds that are known to contribute more to the health benefits that could come from brassica sprouts consumption. The two Portuguese brassica varieties studied, Galega kale and Penca cabbage, showed to be good source of aliphatic-GLs. However, the losses during storage in this group of GLs was very high, especially in Galega kale. WS sprouts showed always a more stable Aliphatic-GL content during the first days of storage. Different contents of hydroxycinnamic acids were detected in the Brassica sprouts during storage, with the WS being particularly rich in these bioactive compounds. Long storage periods tend to reduce the phenolic acids. Anthocyanins were only observed in red cabbage sprouts which were particularly rich in malvidin-3-glucoside. Sprouts, even when submitted to long refrigerated storage periods could be considered safe food products from the perspective of their microbiological and biogenic amine levels. However, efforts should be made to establish their level of acceptance based in this chemical markers.

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CAPÍTULO 8

Considerações finais

Considerações finais

A obtenção de informação relevante sobre a qualidade de germinados de *Brassica oleracea* foi uma das preocupações que esteve sempre na base do desenvolvimento do trabalho apresentado nesta tese. Pretendia-se principalmente valorizar um novo produto alimentar, os germinados, confirmando as suas mais-valias nutricionais, e também determinar a potencialidade das variedades tradicionais portuguesas, a couve-galega e couve-penca para a produção de germinados. Para isso, o trabalho desenvolvido procurou caracterizar a composição nutricional e identificar e quantificar os compostos bioactivos presentes em germinados de quatro variedades de *B. oleracea* (brócolo, couve-roxa, couve-galega e couve-penca). Procurou-se também relacionar a qualidade dos germinados com as condições ambientais fornecidas durante a germinação (presença de luz e duração do período de germinação), para encontrar a relação que potencializasse ao máximo as qualidade nutricionais deste produto. Desta forma, pretendia-se obter informação relevante para que os produtores consigam um produto de maior valor económico e que atraia os novos consumidores. Essa informação é ainda mais importante no caso das variedades portuguesas, uma vez que poderá valorizar ainda mais um produto de origem tradicional e dar-lhe uma nova forma de consumo, estimulando a sua produção e consumo nos mercados locais.

Como produtos vegetais prontos a consumir, a qualidade nutricional destes produtos está directamente relacionada com a presença de compostos bioactivos que possam contribuir para a melhoria ou manutenção do estado de saúde dos consumidores. Neste sentido, procurou-se apresentar neste trabalho a composição destas quatro variedades e perceber as mais-valias deste produto. Desta forma, é possível apontar como principais vantagens da composição dos germinados os seguintes aspectos:

- A elevada percentagem de proteína (23-31 g/100 g (peso seco) e fibra dietética (25-38 g/100g (peso seco) que todos os germinados apresentaram; um maior teor de minerais que os vegetais maturos, em que o teor de selénio se destacou, representando umas das grandes mais valias da composição destes produtos; o perfil de aminoácidos destes produtos mostrou também ser bastante equilibrado, possuindo alguns aminoácidos essenciais na composição como a treonina, valina, fenilalanina, isoleucina e leucina.

- A composição em glucosinolatos dos germinados é sem dúvida uma das grandes vantagens nutricionais destes produtos, uma vez que são normalmente consumidos crus o que permite uma maior preservação destes compostos; os germinados estudados mostraram um elevado teor de glucosinolatos alifáticos, como

a sinigrina e a glucorafanina, reconhecidos pela sua potencial ação anticancerígena; das variedades estudadas, os germinados de couve-galega destacaram-se dos demais devido ao seu elevado teor de glucosinatos alifáticos, especialmente devido ao seu teor de sinigrina.

- A presença de compostos com reconhecida actividade antioxidante foi também uma das mais-valias presentes na composição dos germinados, cujo perfil de compostos fenólicos foi caracterizado pela predominância de ácidos hidroxicinámicos, principalmente compostos derivados de ácido sinápico.

- A composição em ácidos orgânicos mostrou uma grande predominância do ácido cítrico e málico na composição dos germinados. Contudo, a presença de ácido oxálico também se destacou na composição destes, sendo este composto reconhecido por interferir na absorção de cálcio e outros minerais se ingeridos em elevadas quantidades.

- A composição dos germinados revelou também possuir uma potencial actividade antimicrobiana contra alguns dos patogénicos mais preocupantes no que diz respeito à segurança dos produtos alimentares. Esta actividade mostrou também uma elevada correlação com alguns dos ácidos orgânicos e fenólicos encontrados na sua composição.

Apesar de na maioria dos casos, os teores de compostos encontrados nos diferentes germinados se encontrarem dentro de gamas muito semelhantes, a sua composição demonstrou também uma influência clara da informação genética presente em cada variedade. Esta traduziu-se principalmente pela dominância de determinados glucosinolatos e ácidos orgânicos na composição de cada variedade, e também pela forma como cada variedade reagiu às condições ambientais a que foi submetida. Relativamente às condições ambientais testadas e à influencia do tempo de germinação na composição dos germinados, as principais conclusões que foram retiradas deste trabalho foram:

-A ausência de luz durante a germinação potenciou um perfil de aminoácidos e de ácidos gordos mais equilibrado, porém o exposto pareceu produzir germinados com maior teor de selénio.

- A análise da potencial ação antioxidante dos germinados apresentou valores superiores nos germinados expostos a ciclos de luz/escuro. Este foi também o fotoperíodo que mais potenciou o teor de glucosinolatos e de alguns ácidos orgânicos encontrados nas variedades estudadas.

- Quanto ao período de germinação, na maioria dos compostos estudados, o uso de períodos de germinação mais curtos (entre 7 e 9 dias) originou a presença de um maior teor de glucosinolatos e alguns compostos fenólicos.

Como este é um produto minimamente processado, o seu tempo de vida útil, qualidade microbiológica e também a estabilidade da sua qualidade nutricional, até chegar ao consumir, são factores muito importantes para a aceitação destes produtos no mercado. Neste sentido, o trabalho apresentado nesta tese pode também comprovar que a qualidade das variedades de germinados estudadas beneficiam de um período de refrigeração inferior a 7 dias, apresentando as amostras germinadas no escuro uma maior estabilidade do teor dos compostos bioactivos. Em termos microbiológicos, as amostras estudadas foram consideradas como seguras durante os doze dias de armazenamento.

Como perspetivas futuras, é fundamental continuar os estudos de caraterização de germinados de diferentes espécies hortícolas, e adaptar a tecnologia existente à produção de germinados de espécies hortícolas portuguesas, em particular a partir de variedades regionais, uma vez que poderá permitir o desenvolvimento de novos produtos, com valor acrescentado, distintos, adaptados ao consumidor nacional e passíveis de serem patenteados. Além disso, as condições de conservação pós-colheita variam de forma apreciável entre os diferentes tipos de germinados, o que requer investigação aprofundada, para adaptar a tecnologia já existente a estes novos produtos, desenvolvidos a partir de variedades portuguesas. O estudo das condições de conservação (embalagem para produto em fresco e produto em conserva) ajustadas a estes produtos permitirá ainda dar resposta às necessidades de mercado, ao nível dos diferentes canais de comercialização, oferecendo um produto seguro e saudável.